

Studies relating to the Breast, its Tumours and Fluids

by

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Declaration

I certify that the publications contained in this thesis have not been previously submitted for any higher degree. The work contained in the publications in which I am first author was carried out by me or under my immediate supervision. In the case of publications in which I am not first author I declare that I have made a substantial and significant contribution to the work as described in the "Explanation of Coauthorship".

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ABSTRACT OF THESIS (Regulation 7.9)

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No. of words in the main text of Thesis

This thesis comprises 61 papers which describe work undertaken between 1972 and 1985. The research was performed in order to obtain basic information on the environment and the biochemical processes existing within the female breast. It was thereby hoped to achieve a better understanding of events which occur during the development of breast disease, particularly breast cancer.

Hormones are involved not only in the natural development of normal breast but also in the aetiology of many breast abnormalities and, most importantly, in the continued growth of a proportion of breast cancers. A major aspect of the research described in this thesis is concerned with the measurement of steroid hormones, their metabolism and receptors in the breast, its tumours and secretions. Additionally studies have been performed to assess the significance of other markers of tumour behaviour and to determine whether agents such as LHRH analogues have direct effects within the breast.

The potential of the breast to modify its own hormonal environment has been investigated by performing in vitro incubations with steroid precursors. Pathways leading to both active androgens and oestrogens have been identified. Biosynthesis of 5 α reduced androgens occurs in all types of breast tissue but is particularly associated with apocrine differentiation. The production of oestrogen or "aromatization" was detected in a proportion of breast cancers but not in non-malignant tissue. The significance of tumour aromatase is controversial but it may be important in oestrogen-dependent cancers growing in post-menopausal women who have low circulating levels of oestrogen.

In order to estimate levels of androgen precursors within the breast, measurements of DHA sulphate have been made in breast secretions obtained by nipple aspiration and breast cyst fluids. Remarkably high, but variable, concentrations have been detected. Further investigations of the composition of such breast fluids have shown them to have a distinctive composition in terms of ionic content, major types of immunoglobulins and concentrations of plasma-, platelet-associated and other proteins. Cyst fluids may be subdivided into two major populations which are lined by different epithelium and have a differing natural history.

Several series of breast cancers have been analysed for either androgen receptors, progesterone receptors, cyclic AMP binding proteins, prostaglandins or expression of lectin binding. These have been suggested to be markers of metastatic potential or hormone-responsiveness. Inter-relationships have been made with oestrogen receptors and other tumour/patient characteristics but assessment of clinical value awaits patient follow-up.

The possibility that polypeptide hormones have direct actions on the breast was investigated by culturing breast cancer cells with LHRH and its analogues. Marked inhibitory effects which appear to be mediated by a specific recognition mechanism have been demonstrated. These findings are reviewed in terms of their significance to the management of patients with breast disease.

Abstract

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Acknowledgement

This thesis would not have been possible without the help of a whole series of people including my colleagues, technicians and friends both inside and outside the University Department of Clinical Surgery. That this is so can be deduced from the Introduction to this thesis and from the coauthorship and acknowledgements in the publications. I hope that these will serve to document my thanks and provide evidence of the teamwork which has been essential to accomplish this research. When, after offering gratitude to a team, individuals are selected to special mention, there is a risk of unwittingly demeaning the contribution of the unmentioned. By specially alluding to this, I hope I have avoided the problem. I, therefore, wish to offer special thanks to Professor Sir Patrick Forrest in whose department this work was largely performed and who has been a constant source of support and stimulating advice. Additionally, my gratitude goes to Miss J Telford, who has acted as my right-hand woman in the laboratory, taken the "blame" when things have gone wrong without seeking reward when things have gone right and providing the technical continuity which is essential for good research. Last of all, I have to acknowledge the support of my family who have put up with this bad-tempered man and who have been the motivation behind the writing of this thesis. I would, therefore, dedicate this thesis to my wife, Janet and the boys, Neil and Mark.

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Explanation of Joint Authorship

Those publications in which I am first author results from work carried out by me or under my direct supervision and the paper was written by myself in each case.

With regard to publications in which I am not the first author, I include the following details of my contributions:

Publications 9 and 13: The work was performed under my supervision by Mr. R.C. Mason who was a M.R.C. clinical research fellow in my laboratories. I also wrote sections of the papers and revised the final manuscripts before publication.

Publications 20 and 21: This work was performed under my supervision by my Ph.D. student, Mr. P. Buchan, on mammary tumours from animals for which I was responsible. Mr. Buchan and I together wrote the manuscripts.

Publication 31: I performed the measurements of aetiocholandone in urine specimens from patients under the care of Mr. T. Hamilton. Analysis of results was carried out by Dr. R. Prescott who also wrote most of the paper. I wrote the section of the methods relating to urinary analysis and contributed to the appropriate part of the discussion.

Publications 34 and 43: Analyses were performed by Dr P-L Yap on breast fluids supplied and characterized by myself. The papers were written by Dr. Yap but I was heavily involved in their revision.

Publications 38, 39, 40, 41, 42 and 46. These papers were primarily written by Mr. J.M. Dixon who was a clinical research fellow in my laboratory. He also obtained the clinical and pathological data. I carried out the biochemical measurements, wrote aspects of the paper and made the secondary revisions of the manuscripts.

Publications 47, 48 and 49: Dr. R.A. Hawkins performed the oestrogen receptor measurements and wrote the papers. I provided the mammary tumours which were analysed and carried out the endocrine manipulations on the animals under study. I was also involved in the revision of the manuscript.

Publication 50: This paper was written by my clinical research fellow, Mr. R.C. Mason. The oestrogen receptor measurements were performed by Dr. R.A. Hawkins but I was responsible for the determination of DNA content and also made a contribution to the writing of the paper.

Publication 53: My research associate, Mrs. D.M.A. Watson, carried out the measurements of prostaglandins under the supervision of Dr. R.W. Kelly. I designed the study protocol and initiated the work. The paper was written jointly by Mrs. Watson and myself and I was responsible for revising the manuscript into its final published form.

Publication 56: I personally performed the measurements of cyst protein and provided the appropriate figures for the paper.

Publication 57: Dr. R.A. Walker carried out the measurements of lectin binding upon material supplied by myself and also wrote the paper. I assayed the tumours for progesterone receptors and revised the manuscript.

Publication 60: This review was written conjointly by Sir Patrick Forrest and myself.

Introduction

Cancer of the breast is the most common female malignancy in the United Kingdom, now affecting one in every fifteen women in Scotland. It is a disease which appears to metastasize early in its natural history and, once disseminated, the outlook is bleak. Survival rates from breast cancer have not significantly improved over the last 50 years.

Although not having either the immediate life-threatening potential or emotive implication of breast cancer, the group of conditions collectively known as "benign breast disease" represent the most common breast problem which will face the clinician. These abnormalities create severe anxiety in many women who fear the presence of cancer or a predisposition to malignancy. This is particularly so for breast cysts which appear as lumps within the breast and affect about 8% of women. Although cysts may be simply treated by needle aspiration, many refill or recur which results in repeated treatment and places a heavy burden on clinical time.

There is therefore considerable pressure to understand the factors which influence the initiation and development of both malignant and benign diseases of the breast. It is my personal opinion that major progress to eradicate these conditions will only come from fundamental research into the biochemical and molecular events occurring locally within the breast. The results presented within this thesis reflect this philosophy.

Section 1 describes the investigations performed to assess the potential of human breast and its tumours to synthesize active steroid hormones. It has long been known that steroids are

intimately involved not only in the natural development of normal breast but also the aetiology of breast abnormalities, notably the induction and maintenance of growth of a proportion of breast cancers. Whilst, in premenopausal women, the ovaries are the primary site of steroid production, after the menopause peripheral biosynthesis in non-endocrine tissues becomes more important. The ability of the breast to modify its own environment of active steroid hormones has therefore been explored by performing in vitro incubations of breast tissues with radioactively-labelled precursors. Early studies^(1,2) showed that normal, benign and cancerous female breast were all able to metabolize androgen precursors. Conversions included the transformation of dehydroepiandrosterone (DHA) to testosterone and testosterone to Δ^4 androstenedione, 5α dihydrotestosterone and 5α androstane- 3β -diol. Gynaecomastic male breast tissue also performed some, but not all, of these steroid conversions⁽³⁾. The report in reference (4) that a breast cancer could synthesize oestradiol from testosterone was the first unequivocal evidence for oestrogen biosynthesis or "aromatization" by human breast tissue. Until this time, it had been thought that oestrogen biosynthesis was restricted to the endocrine glands of the gonads and foetal-placental unit but it is now recognised that at least one half of breast cancers possess the activity^(5,6). Aromatization by breast cancers appears to be inhibited by classical inhibitors of the system such as aminoglutethimide⁽⁷⁾ and tends to be associated with oestrogen receptor-positive tumours although the correlation is not absolute^(8,9). Local oestrogen production in tumours may have

clinical significance if levels synthesized are sufficient to stimulate oestrogen-dependent growth. Such tumours would be expected to respond to therapies which inhibit tumour aromatase and there is evidence that this is the case^(10,11). Efforts have also been made to determine factors influencing steroidogenesis in breast tumours. In vitro addition of oestrogen to incubates of breast cancer consistently inhibited metabolism of testosterone to its 5 α reduced products and Δ^4 androstenedione⁽¹²⁾. Although hyperprolactinaemia influences 5 α reduction of androgens in rat mammary tumours (see Section B), no effects were demonstrable on any aspect of androgen metabolism in human breast tissue⁽¹³⁾. However, human breast cancers in which apocrine characteristics were a marked feature metabolised significantly more testosterone by 5 α reduction than those in which apocrine features did not predominate⁽¹⁴⁾. All these aspects of steroid metabolism by human breast tissues have been reviewed^(15,16).

In order to investigate more extensively the effects of hormone manipulation on steroidogenesis by breast tissue it was important to develop an experimental animal model. Studies were therefore performed with dimethylbenzanthracene (DMBA)-induced rat mammary carcinomata (section B). These tumours were shown to have a similar spectrum of steroid transformations as human breast cancer except that oestrogen biosynthesis was not demonstrated⁽¹⁷⁾. The growth of many carcinogen-induced mammary tumours are hormone-dependent and, in particular, may be stimulated by prolactin. It was thus of interest that hyperprolactinaemia induced in rats either before or after tumour appearance significantly increased 5 α

reduction of testosterone^(18,20) in the cancers. These effects of hyperprolactinaemia on growth and tumour 5 α reduction were markedly decreased by ovariectomizing the animals⁽²¹⁾. Ovariectomy itself significantly stimulated 5 α reduction of testosterone by rat mammary carcinomata, an effect which was reversed by administering oestrogen to the animals⁽²²⁾. Effects were also observed after in vitro addition of either oestrogen⁽²³⁾ or prolactin⁽²⁴⁾ to incubates of DMBA-induced tumours. Comparative studies were also performed with rat mammary tumours which, although originally induced by DMBA, had been transplanted into successive generations of host animals⁽²⁵⁾. Whilst, at early passages, the tumours were hormone dependent and regressed after ovariectomy, later tumour generations appeared ovary-independent⁽²⁶⁾. This change in hormone-sensitivity was associated with a progressive increase in tumour metabolism of testosterone to 5 α androstane diols⁽²⁶⁾. It was therefore of interest that endocrine ablation treatment of animals bearing multiple transplanted tumour caused increased 5 α reduction, an effect which could be reversed by oestrogen replacement⁽²⁷⁾.

For comparative purposes, studies were also performed to determine the pattern of steroid metabolism in other human tissues. These included normal, benign and malignant thyroid⁽²⁸⁾ and metastatic deposits of a bronchogenic carcinoma⁽²⁹⁾.

The impetus to investigate androgen metabolism by breast tissues had come from the pioneering work of Dr. R.D. Bulbrook of the Imperial Cancer Research Fund who had suggested that urinary and plasma androgens of women with breast cancer and those who would subsequently develop the disease were subnormal in comparison with

control women. These results prompted studies which it was hoped would produce confirmatory findings in a population of women from South-East Scotland (Section C). In the event, these investigations failed to detect any significant differences between urinary androgens and established prognostic factors measurable at the time of primary treatment for breast cancer⁽³⁰⁾ or, indeed, time to recurrence and survival in these patients⁽³¹⁾. Nevertheless, a by-product of these investigations was that a radioimmunoassay had been established to measure the androgen conjugate DHA-sulphate in circulating plasma. Furthermore, my attention had been drawn to the work of Dr. O. Sartorius in California who had obtained breast secretions from non-lactating women by nipple aspiration. Whilst these fluids had been obtained, as a diagnostic tool, to screen for the presence of malignant cells, it occurred to me that the secretions might better reflect the steroid environment within the breast than measurements of urinary or plasma hormones.

I therefore embarked upon a project to measure DHA-sulphate in breast fluids which subsequently expanded into a major interest in the composition of both breast secretions and cyst fluids. The principle findings are described in Section D. Breast secretions obtained by nipple aspiration from non-lactating women contained remarkably high levels of DHA-sulphate⁽³²⁾. The mean concentration was over 200 fold higher than in circulating plasma. G.l.c.-mass spectrometric analysis performed in collaboration with Dr. R.W. Kelly, MRC Unit of Reproductive Biology, confirmed that the material cross-reacting within the radioimmunoassay was a conjugate of DHA⁽³²⁾. Although the levels of DHA-sulphate in breast secretions

varied greatly between fluids obtained from different women, concentrations were relatively constant within individual women in terms of different ducts in the same breast, different breasts of the same individual and sequential samples from the same women⁽³³⁾. No significant difference was detected between levels in breast secretions obtained from normal women and those in patients with early malignant or benign breast disease⁽³³⁾. However, comparative data on secretions from tumour- and non-tumour-bearing breast from the same patients are limited and, interestingly, in contrast to women with breast disease, there is a tendency for greater variation between the breasts of individuals with breast cancer⁽³³⁾. The physiological significance of such large concentrations of hormone within the breast remains to be determined. Although DHA-sulphate has little biological activity per se, the metabolic activity of the breast, as described in Section A, means that it may be transformed into active hormones which are capable of modifying breast development. Other studies have been performed in collaboration with Dr. P.L. Yap, Blood Transfusion Service, on the protein composition. These showed that breast secretions may contain measurable levels of immunoglobulins A and G (IgG and IgA), lactoferrin and lysozyme⁽³⁴⁾. The IgA was characterised as the secretory 11S form which suggests that a secretory immune system may be functional in the non-lactating breast⁽³⁴⁾. In addition to breast secretions, fluids aspirated from breast cysts have been studied. These also may contain remarkably high concentrations of DHA-sulphate which are higher than those in circulating plasma but somewhat lower than in breast secretions⁽³⁵⁾.

A feature of DHA-sulphate levels in breast cyst fluids was the wide variation in concentrations between different cysts. Levels were not related to age, parity or menopausal status of the patients or the volume of cyst fluid aspirated^(35,36). In patients with multiple cysts, values for DHA-sulphate in cysts aspirated from the same breast on the same occasion were relatively comparable but wide differences sometimes existed between cysts aspirated on different occasions from the same breast⁽³⁶⁾. These observations have led to more extensive studies on the composition of cyst fluids. An important finding was that the concentrations of Na⁺ and K⁺ varied greatly between different cyst fluids and were related to levels of DHA-sulphate⁽³⁷⁾. Furthermore, the distribution of values for Na⁺ and K⁺ was suggestive of the existence of more than a single population of cyst fluids. As a result, cysts are now routinely classified according to their electrolyte and androgen conjugate composition⁽³⁷⁾. The classification appears to relate to the natural history of cystic disease and, perhaps, subsequent risk of breast cancer. Thus, work in collaboration with Mr. J.M. Dixon has shown that cysts may be lined either by classical apocrine or "flattened" epithelium and that electrolyte content related absolutely with these histological types⁽³⁸⁾. Cyst lined by apocrine epithelium and possessing fluids with high K⁺ and relatively low Na⁺ (Type I) are significantly more likely to be multiple and recurrent than those with flattened epithelium and the converse electrolyte composition (Type II)^(39,40). Retrospective analysis of patients with breast cancer who had had a previous cyst aspiration which could be typed histologically or by electrolyte

composition showed that these were likely to be Type I, suggesting that apocrine change occurs more frequently in populations at risk of breast cancer^(39,40). The pH and protein composition of cyst fluid also vary widely^(42,43) and this variation can largely be accounted for by different subpopulations of cysts. Thus Type I fluids have a low pH and the secretory form of IgA whilst Type II have a higher pH and the non-secretory form of IgA⁽⁴⁴⁾. These results have led me to suggest that Type I cysts are formed by active apocrine secretion whereas Type II cysts occur by a more passive process⁽⁴⁴⁾. This theory has had to be modified recently following the finding that Group II cysts have higher concentrations of thrombospondin, a platelet-associated protein, than occurs either in Type I cysts or the circulation⁽⁴⁵⁾. This would indicate that some form of active process is required to account for the accumulation of this particular constituent⁽⁴⁵⁾. These studies on the composition of breast secretions and cyst fluids have led to the suggestion that DHA-sulphate may be a useful marker of apocrine secretion within the breast ⁽⁴⁴⁾ - the steroid conjugate is also found in very high concentrations in microcysts which are lined by apocrine epithelium ⁽⁴⁶⁾ and may be detected by immunohistochemistry in breast cancers which display apocrine features⁽⁴⁴⁾.

I have also been interested in the measurement of biochemical markers which might predict for the prognosis and behaviour of breast cancer in terms of aggressiveness, hormone-dependence and potential for metastatic spread (Section E). Part of the problem in assessing the value of markers, particularly in the prognosis of early breast cancer, is the relatively long interval before many

patients present with recurrent disease and hence the need for an extended follow-up period. However, a quicker guide to the potential of tumour markers may be obtained by analysing relationships with established factors of prognostic value. In breast cancer, these include tumour involvement of axillary lymph nodes, tumour size, grade and stage and the most recently validated factor of tumour oestrogen receptor status. In this respect, I have been very fortunate to have as a colleague Dr. R.A. Hawkins, who established a routine assay for the measurement of oestrogen receptors. This was originally used to assess the effects of endocrine manipulation and hormone responsiveness in rat mammary tumours⁽⁴⁷⁻⁴⁹⁾. Whilst the value of oestrogen receptors in predicting hormone sensitivity of breast cancers, particularly when accurately corrected for cellularity⁽⁵⁰⁾, is recognised, the receptors for androgens in breast tumours have been less extensively investigated. I, therefore, established an assay for androgen receptors which could be used routinely for measurements in breast cancers⁽⁵¹⁾. Results showed that whilst androgen receptor activity was not related to lymph-node involvement with tumour, there were significant positive correlations with the presence of both oestrogen receptors and progestogen receptors⁽⁵²⁾. Prostaglandins have been implicated in the metastatic spread of breast cancer, particularly in bone. Although breast tumours appear to produce prostaglandin-like material, most investigators have employed bioassay or radioimmunoassay techniques which do not definitively identify prostaglandins. I, therefore, instigated a study performed by my research associate, Mrs. D.M.A. Watson, in

collaboration with Dr. R.W. Kelly, in which prostaglandins were measured by gas liquid chromatography - mass spectrometry in extracts of breast cancers. Definitive evidence was obtained for the presence of varying amounts of both prostaglandin E_2 and $F_2\alpha$ in these extracts⁽⁵³⁾. Whilst concentrations of the prostaglandins were significantly related to each other, levels were not correlated with the presence of oestrogen and progesterone receptors⁽⁵³⁾. An assay has also been set up and validated by which to measure cyclic AMP binding proteins in breast cancers⁽⁵⁴⁾. As with the results on androgen receptors and prostaglandins, the full value of these measurements is awaiting clinical follow-up of the patients. Other tumour markers which have been studied are carcinoembryonic antigen⁽⁵⁵⁾, various milk proteins⁽⁵⁶⁾ and lectin binding sites⁽⁵⁷⁾. Whilst the association of steroid hormones with breast disease has been extensively studied, the involvement of polypeptide hormones has been relatively ignored. My interest in polypeptides and the breast was awakened by a discussion with Dr. H.M. Fraser, MRC Unit of Reproductive Biology who had long had an interest in luteinizing hormone-releasing hormone (LHRH) and its analogues. Agonist analogues of LHRH had been used to treat premenopausal patients with advanced breast cancer using the rationale that chronic administration would perturb the pituitary-ovarian axis and produce a medical castration. The possibility that LHRH and its analogues could have direct anti-tumour effects had not been explored. We, therefore, decided to test LHRH analogues against human breast cancer cell lines maintained in culture (Section F). As a result we obtained the first unequivocal evidence that an LHRH agonist was

capable of major direct inhibitory effects on the growth of breast cancer cells⁽⁵⁸⁾. These effects appear to be mediated by a specific recognition mechanism, being reversed by an LHRH antagonist⁽⁵⁸⁾. Such an action would account for the beneficial effects reported in postmenopausal women with breast cancer treated with LHRH agonists and has important implications with regard to future therapy. Studies are continuing to elucidate more fully the mechanism of action of LHRH analogues and the susceptibility of different breast cancer cell types to their action⁽⁵⁹⁾.

The results and concepts presented in this thesis have also formed the basis of reviews on hormones and the breast written as co-author with Professor Sir Patrick Forrest⁽⁶⁰⁾ and Dr. T.A. Anderson (Section G).

VII PUBLICATIONS

SECTION A

STEROID METABOLISM BY
THE HUMAN BREAST AND ITS TUMOURS

METABOLISM OF ANDROGENS BY HUMAN BREAST TISSUE

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Summary

Evidence for the conversion of dehydroepiandrosterone through testosterone to 5 α -dihydrotestosterone has been sought in three breast cancers, two fibroadenomas, one specimen of fibroadenosis, and six specimens of normal breast tissue from the same patients. Significant in-vitro activity was demonstrated in all tissues.

Introduction

HUMAN breast cancer possesses the enzymes necessary for steroid biosynthesis.^{1,2} Incubations and perfusion with various precursor steroids has indicated that a whole range of conversions may occur.^{3,4} Jenkins and Ash⁵ reported that the conversion of testosterone to 5 α -dihydrotestosterone was apparently limited to cancerous tissue and did not occur in the normal surrounding breast. The study we report was also performed to determine the specificity of this activity in breast cancer. Two transformations, dehydroepiandrosterone (D.H.A.) to testosterone and testosterone to 5 α -dihydrotestosterone, have been assessed in samples of cancer, fibroadenoma, and fibroadenosis of the human breast, and in normal breast tissue from the same patients.

Methods

Tissue was obtained from three patients with breast cancer, two with fibroadenomas, and one with fibroadenosis.

In all the patients, samples were taken both of the lesion and of the normal breast tissue. Histological examination of all tissues was carried out initially by frozen section and, when further studies were required, by embedding in paraffin. Particular care was taken to exclude neoplastic infiltration in the normal breast tissue taken from the cancer patients.

The specimens were finely sliced and incubated for two hours at 37°C in Krebs-Ringer phosphate buffer (pH 7.4) (10 ml. per g. tissue) containing an N.A.D.P.H.-generating system, 20 μ Ci tritiated 7 α -D.H.A., and 2 μ Ci (4-¹⁴C) testosterone. The conversions of D.H.A. to testosterone and testosterone to 5 α -dihydrotestosterone were determined by measuring the percentage incorporation of radioactive labels into the metabolites, which were extracted and purified by thin-layer chromatography. Details of the methods are described elsewhere.^{3,6} The total D.N.A. content per gramme of wet tissue was estimated in each specimen by the method of Burton.⁷

Results

The percentage radioactivity found in the relevant metabolites is shown in the accompanying table. Both benign and malignant tumours transformed D.H.A. to testosterone. There were no consistent differences in conversion between the groups. The low value for one fibroadenoma (patient 6) can be accounted for by further metabolism of testosterone, since significant ³H-labelling of 5 α -dihydrotestosterone and 5 α -androstadiol was also found.

All samples of normal and neoplastic tissue reduced testosterone to 5 α -dihydrotestosterone, and, in contrast to the conversion of D.H.A. to testosterone, 5 α -reductase activity was markedly increased in the fibroadenomas, and to a lesser extent in the normal breast associated with them, than in the tumours and normal tissues from the other patients.

Discussion

These results indicate that the conversion of D.H.A. to testosterone and testosterone to 5 α -dihydrotestosterone, which is known to occur in cancer of the breast, also occurs in benign tumours and in normal breast tissue. The demonstration that 5 α -reductase was not only present in normal breast tissue but that its activity was quantitatively similar to that in the tumour does not accord with the findings of Jenkins and Ash,⁵ who did not find such activity in the specimens of normal breast which they examined.

METABOLISM OF (7-³H) D.H.A. AND (4-¹⁴C) TESTOSTERONE BY HUMAN BREAST TISSUE

Patient	Age (yr.)	Histology of tumour	% Conversion of:				
			D.N.A. content (mg./g. tissue)		D.H.A. to testosterone		Testosterone to 5 α -dihydrotestosterone
			Tumour	Normal breast	Tumour	Normal breast	
1	40	Undifferentiated carcinoma of large-cell type	1.79	0.84	0.18	0.29	0.40
2	50	Intraduct carcinoma with a small focus of invasion	1.29	0.88	0.16	0.23	0.89
3	50	Carcinoma, possibly apocrine in origin—later produced widespread metastases	3.86	0.59	0.53	0.48	0.06
4	35	Fibroadenosis with duct ectasia	1.93	0.76	0.64	0.59	0.19
5	20	Simple fibroadenoma	2.15	0.94	0.46	0.10	1.72
6	20	Simple fibroadenoma	2.05	0.32	0.07	0.31	8.02

The production of large amounts of 5α -dihydrotestosterone by the two fibroadenomas is especially interesting. This increased activity is unlikely to be caused by greater cellularity of the fibroadenomas. Their D.N.A. content was within the range of that of the carcinomas and their activity in converting D.H.A. to testosterone was no different from that of other tissues. These two patients were taking oral contraceptives, which may have influenced 5α -reductase activity in the breast, since oestrogens affect 5α -reductase in the liver⁸ and adrenal cortex.⁹

Although there were quantitative differences in the different tissues, we conclude that the potential for producing hormones of high biological activity is shared by malignant and benign tumours of the breast and normal breast tissue. Studies are now being carried out to determine whether similar activity can be demonstrated in tumours at other sites.

We thank Mr T. Hamilton, who allowed us to study material from one of his patients, and Miss J. Telford and Mrs A. Boyd for their skilled technical assistance. This work was performed with a grant from the Cancer Research Campaign, from which two of us (W. R. M. and D. McD.) receive full-time support.

Requests for reprints should be addressed to W. R. M.

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Steroidogenesis in Human Breast Cancer, Benign Breast Disease and Normal Breast Tissue

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Human breast cancer can transform certain steroids into physiologically active hormones and thereby act as a paraendocrine organ (Adams & Wong, 1968; Jones *et al.*, 1970). Breast carcinoma thus may have the potential to utilize inactive precursors to produce a local environment of those hormones which, in the experimental animal, have been shown to influence tumour growth. The aim of the present study was to determine whether this potential was shared by human benign breast tumours and normal human breast tissue.

To make the comparison, the following tissues were studied: three breast carcinomas, two fibroadenomas and one specimen of fibroadenosis of the breast. From each patient in which the above tissues were removed, a specimen of associated normal breast tissue which was histologically free from neoplastic change was also examined.

The tissues were finely sliced in Krebs-Ringer phosphate buffer, pH 7.4 (10 ml/g of tissue). An NADPH-generating system (200 μ mol of glucose 6-phosphate, 25 mg of NADP and 50 units of glucose 6-phosphate dehydrogenase) and radioactively labelled steroid precursors (20 μ Ci of [7-³H]dehydroepiandrosterone and 2 μ Ci of [4-¹⁴C]-testosterone) were added. Incubation was immediately carried out at 37°C for 2 h in O₂. The steroid interconversions were determined by measuring the percentage incorporation of the appropriate radioactive label into the individual metabolites after extraction and purification by thin-layer chromatography. Details of the methods of steroid

Table 1. Metabolism of [7-³H]dehydroepiandrosterone by breast tissue
For details see the text.

		% of ³ H radioactivity found in isolated steroids					
Metabolism by carcinoma and associated normal breast tissue		J. C.		A. P.		M. M.	
Patient ...	Tissue ...	Carcinoma	Normal breast	Carcinoma	Normal breast	Carcinoma	Normal breast
Dehydroepiandrosterone Testosterone		75.50	90.50	*	*	65.40	76.20
		0.18	0.29	0.16	0.23	0.53	0.48
Metabolism by benign tissue and associated normal breast tissue		% of ³ H radioactivity found in isolated steroids					
		J. G.		E. H.		E. McG.	
Patient ...	Tissue ...	Fibroadenosis	Normal breast	Fibroadenoma	Normal breast	Fibroadenoma	Normal breast
Dehydroepiandrosterone Testosterone		65.70	90.40	39.70	86.60	55.60	66.20
		0.64	0.59	0.46	0.10	0.07	0.31

* Fraction was lost.

Table 2. *Metabolism of [4-¹⁴C]testosterone by breast tissue*

For details see the text.

Metabolism by carcinoma and associated normal breast tissue

% of ¹⁴C radioactivity found in isolated steroids

Patient ...	J. C.			A. P.			M. M.		
Tissue ...	Carcinoma		Normal breast	Carcinoma		Normal breast	Carcinoma		Normal breast
Testosterone	89.80		79.90	87.10		73.60	95.45		98.50
5 α -Dihydrotestosterone	0.40		0.50	0.83		0.89	2.62		0.06
16 α -Hydroxytestosterone	0.017		0.014	0.012		0.011	0.010		0.00

Metabolism by benign tissue and associated normal breast tissue

% of ¹⁴C radioactivity found in isolated steroids

Patient ...	J. G.			E. H.			E. McG.		
Tissue ...	Fibroadenosis		Normal breast	Fibroadenoma		Normal breast	Fibroadenoma		Normal breast
Testosterone	87.43		95.20	22.96		95.50	2.21		73.20
5 α -Dihydrotestosterone	0.25		0.19	20.25		1.72	48.47		8.02
16 α -Hydroxytestosterone	0.00		0.00	0.07		0.04	0.02		0.02

purification and characterization have been described by Fahmy *et al.* (1968) and Jones *et al.* (1970).

The results from these incubations are presented in Tables 1 and 2. As shown in Table 1, a significant amount of dehydroepiandrosterone was metabolized by both malignant and benign neoplastic tissue and also by the normal tissue associated with each. Nevertheless, a large proportion of the precursor remained unmetabolized in each incubation, particularly in the carcinoma and associated-tissue incubations. Testosterone was a metabolite of dehydroepiandrosterone in all incubations, the conversion being of a similar magnitude in normal, benign and malignant tissue; the low value obtained in one fibroadenoma (E. McG.) was probably caused by further metabolism of testosterone, as 5 α -dihydrotestosterone and 5 α -androstenediol were significantly labelled with ^3H .

The metabolism of testosterone by the breast tissues is shown in Table 2. A significant amount of testosterone was metabolized by each type of breast tissue, but, with the exception of the fibroadenoma incubations, a high proportion of the precursor remained unmetabolized. 5 α -Dihydrotestosterone was a metabolite in each incubation; the conversion was much greater in the incubations of fibroadenoma and associated normal tissue than in those of carcinoma and fibroadenosis. Small amounts of 16 α -hydroxy-testosterone were formed from testosterone by the carcinoma and fibroadenoma but not by the fibroadenosis and associated-normal-breast-tissue incubations. In no incubation was oestrogen produced from either dehydroepiandrosterone or testosterone.

These results show that those steroid interconversions which have previously been demonstrated in human breast carcinoma are also present in benign and normal breast tissue. All types of breast tissue investigated possessed 5 α -reductase, $\Delta 5$ -3 β -hydroxy steroid dehydrogenase, 17 β -hydroxy steroid dehydrogenase and 16 α -hydroxylase enzyme activities. Quantitative differences in steroid metabolism were apparent between different breast tissues, the two fibroadenomas studied showing greater activity (particularly in the 5 α -reductase enzyme activity converting testosterone into 5 α -dihydrotestosterone) than the carcinomas. The two patients with fibroadenomas were, however, taking oral steroid contraceptives which are known to influence 5 α -reductase activity in the liver (Schriefers, 1967) and adrenal cortex (Kitay *et al.*, 1970).

The demonstration of 5 α -reductase activity in normal tissue is in contrast to the finding of Jenkins & Ash (1972), who could not show such activity in normal breast tissue obtained at mastectomy from patients with breast cancer. In the present study the activity in normal breast quantitatively reflected the corresponding activity of the associated neoplastic tissue and was low in breasts containing cancers and high in those with fibroadenoma. The conversion of testosterone into 5 α -dihydrotestosterone by breast tissue and, in particular, the high conversion rate in fibroadenomas is of considerable interest in view of the high androgenic activity of 5 α -dihydrotestosterone in the prostate (Bruchovski & Wilson, 1968) and its inhibitory action on oestradiol-dependent rat mammary fibroadenomas (Huggins & Mainzer, 1957).

In summary, it has been shown that human breast carcinoma, human benign breast tissue and normal human breast tissue all have the potential to convert inactive steroid precursors into hormones with high biological activity. The further specificity of these conversions and their effects on the breast and its tumours are still to be determined.

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ANDROGEN METABOLISM IN GYNAECOMASTIC BREAST TISSUE

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SUMMARY

The metabolism of [7α - ^3H]dehydroepiandrosterone and [4 - ^{14}C]testosterone by gynaecomastic breast tissue has been investigated and compared with that in female breast tissue. No conversion of dehydroepiandrosterone to testosterone or testosterone to 5α -androstadiol was detected in gynaecomastic tissue in contrast to female breast tissue in which such conversions were demonstrable. This suggests that 3β hydroxysteroid dehydrogenase activity is absent in gynaecomastic tissue. The relevance of this deficiency is discussed.

INTRODUCTION

Gynaecomastia often represents a symptom of endocrine imbalance. Disorders of the testis (Hall, 1958; Daly *et al.*, 1963; Treves, 1958), adrenal cortex (Treves, 1958; Staffieri *et al.*, 1949; Chambers, 1949), liver (Hall, 1958; Rupp *et al.*, 1951; Becker *et al.*, 1967), thyroid (Ashkar *et al.*, 1970; Becker *et al.*, 1968; Bercovici & Mauvais-Jarvis, 1972), oestrogen metabolism (Bercovici & Mauvais-Jarvis, 1972; Korenman *et al.*, 1969; Lazarev *et al.*, 1969) and pituitary gonadotrophin production (Hall, 1958; Jull & Dossett, 1964) have been implicated but in a large number of patients its aetiology remains unexplained. Surprisingly, investigation of gynaecomastic tissue itself has been neglected although a tissue factor could be involved in the condition. The demonstration that human female breast tissue has the potential to synthesize biologically active androgens (Miller *et al.*, 1973) prompted the present study in which the capacity of gynaecomastic breast tissue for steroidogenesis has been investigated.

MATERIALS AND METHODS

Gynaecomastic patients

Breast tissue was obtained at surgery from six patients. Patient 1 was 31 years old with a 13

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year history of bilateral gynaecomastia. Patient 2 was 21 years old with a 4 year history of right-sided unilateral gynaecomastia. Patient 3 was 24 years old with a 6 month history of left-sided unilateral gynaecomastia and prior to developing gynaecomastia had been receiving phenytoin for 6 months. Patient 4 was 14 years old with a 2 year history of left-sided gynaecomastia. Patient 5 was 64 years old with a 10 week history of right-sided unilateral gynaecomastia. Patient 6 was 35 years old with a 6 week history of left-sided unilateral gynaecomastia. Buccal smear showed the XXY chromosome pattern of the Klinefelter's syndrome.

Histology

Patient 1. Microscopic examination of the tissue from both breasts showed similar appearance which consisted of branching and elongation of the mammary ducts with associated stromal hyperplasia. A few lobular structures with ductules were present but there was no epitheliosis.

Patient 2. The breast tissue showed considerable increase in dense fibrous connective tissue. Intraductal hyperplasia and budding of ducts (some of which contained eosinophilic secretion) was noticeable.

Patient 3. The breast tissue contained dilated ducts embedded in fibrous tissue with evidence of hyperplasia of the lining epithelium and of periductal fibrosis.

Patient 4. Microscopy of representative sections of tissue showed portions of breast tissue

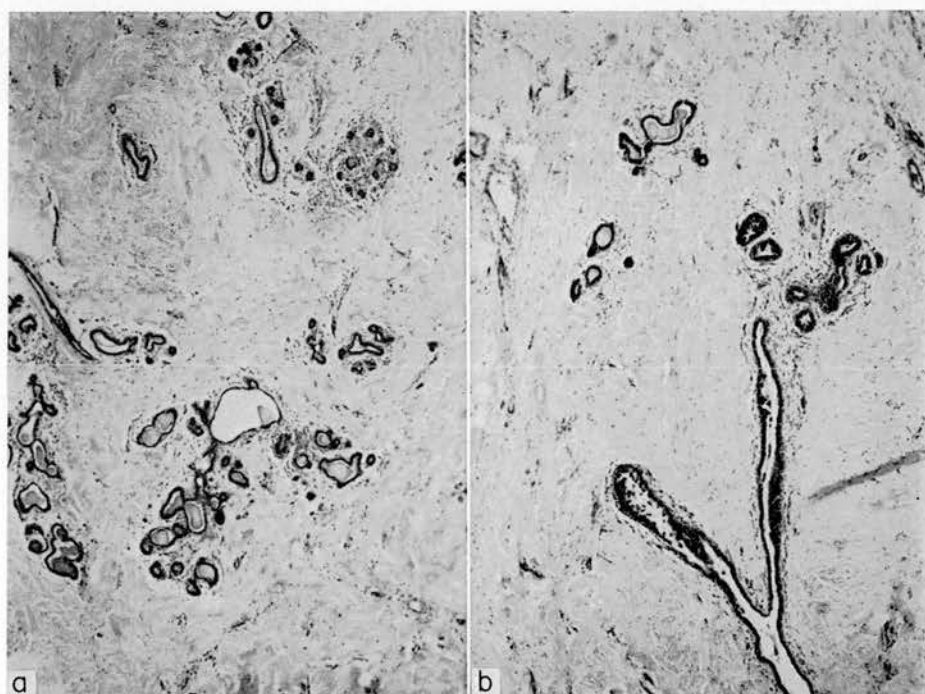


FIG. 1. Representative microscopic sections of breast tissue from patient 4. H. and E. stained magnification, $\times 38$. Note in (a) secretion in ducts and evidence of ductal hyperplasia in addition to stromal hyperplasia.

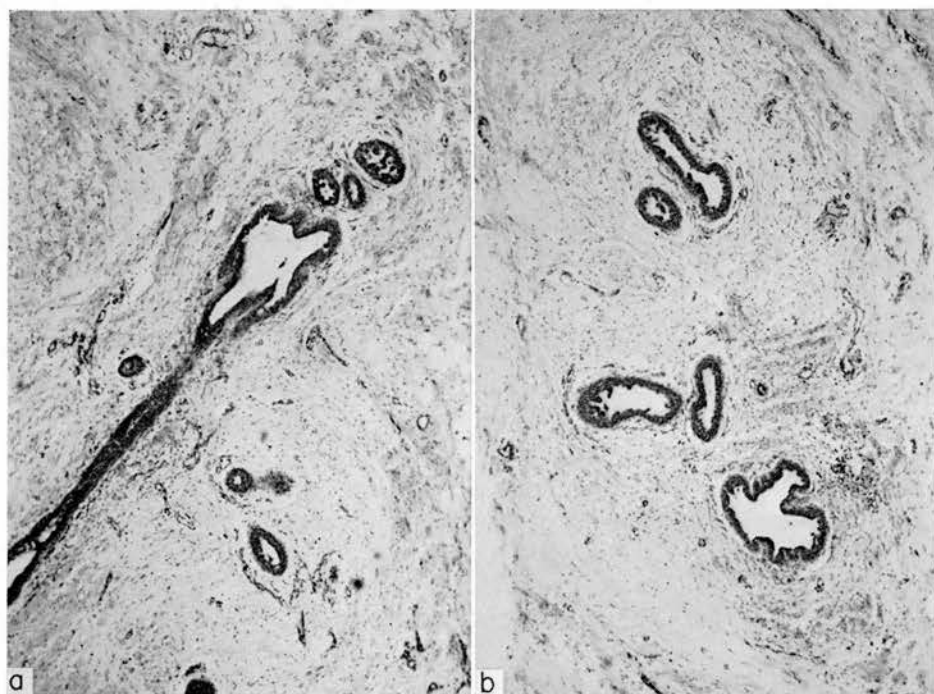


FIG. 2. Representative microscopic sections of breast tissue from patient 5. H. and E. stained magnification, $\times 38$.

with moderately dilated ducts with which some peripheral fibrosis was associated. There was slight tendency to epitheliosis in some of the larger ducts (Fig. 1).

Patient 5. The breast tissue contained dilated ducts with some degree of epitheliosis and was associated with stromal hyperplasia (Fig. 2).

Patient 6. The breast tissue showed marked stromal collagenization with some dilation of mammary ductal epithelium.

Estimations of plasma and urinary gonadotrophin, testosterone and oestrogens, were performed for patients 1, 2 and 4 and found to be within normal limits. In patient 6, however, plasma and urinary gonadotrophins were high, urinary oestrogens low and only urinary and plasma testosterone levels normal. Values for patients 3 and 5 were not available.

Female patients

Patients A and B were 20 years old with simple fibroadenomas of the breast and had been receiving oral contraceptives for some months before tissue biopsy. Patient C was 42 years old with a pericanalicular fibroadenoma and had taken no administered hormones. Patient D was 35 years old with fibroadenosis of the breast with duct ectasia. Patient E was 25 years old with fibrous dysplasia of the breast. Patients F, G and H were 40, 50 and 50 years of age respectively suffering from carcinoma of the breast.

The normal tissue from the carcinoma patients was obtained at mastectomy by dissection from areas of the breast as remote as possible from the primary tumour and the normal

breast associated with the benign condition by biopsy of tissue adjacent to the lesion. Sections of the normal tissue so obtained were examined histologically and found to be free from neoplastic change.

Preparation of tissues and conditions of incubation

All tissues were processed at 0°C until incubation was carried out (within 30 min of tissue removal). The specimens were finely sliced and incubated for 2 hr at 37°C in Krebs-Ringer phosphate buffer, pH 7.4 (10 ml/g tissue) containing an NADPH generating system (200 µmol glucose-6-phosphate, 25 mg NADP and 50 units glucose-6-phosphate dehydrogenase) and the radioactive precursors: 20 µCi [7α-H³]dehydroepiandrosterone (DHA) and 2 µCi [4-¹⁴C]testosterone.

Purification and characterization of metabolites

Before extraction 500 µg of the appropriate non-radioactive carrier steroids were added in ethanol. The metabolites were extracted, purified and separated into individual steroids as described by Fahmy *et al.* (1968). Each purified metabolite was characterized by comparing the specific radioactivities of parent steroids and authenticated derivatives. The procedures for derivative preparation and measurement of cold steroids have been described by Fahmy *et al.* (1968) and Jones *et al.* (1970). Radioactivity was measured by a Nuclear Chicago liquid scintillation counter. Counting conditions were such that ¹⁴C and ³H could be determined simultaneously with efficiencies in Channels I and II respectively at 16% and 52% and 45% and 0%.

TABLE 1. Steroid metabolism by gynaecomastic breast tissue
Metabolism of ³H-DHA

	% radioactive label					
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
DHA	100	99.2	93.8	96.2	95.8	93.7
Testosterone	0	0	0	0	0	0

Metabolism of ¹⁴C-testosterone

	% radioactive label					
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Testosterone	97.10	97.30	96.10	93.40	91.90	95.00
5α-DHT	2.70	0.68	0.40	0.40	0.47	0.26
5α-A'-diol	0	0	0	0	0	0
4-dione	—	—	3.60	0.79	1.74	1.20

5α-A'-diol = 5α-androstanediol; 4-dione = 4-androstenedione.

TABLE 2. Steroid metabolism by female breast tissue

Metabolism of ^3H -DHA		% radioactive label											
		Benign breast						Normal breast					
	Patient A	Patient B	Patient C	Patient D	Patient E	Patient A	Patient B	Patient D	Patient F	Patient G	Patient H		
DHA	39.70	55.60	67.30	65.70	96.40	86.60	66.20	90.40	90.50	—*	76.20		
Testosterone	0.46	0.07	0.19	0.64	0.51	0.10	0.31	0.59	0.29	0.23	0.48		
Metabolism of ^{14}C -testosterone		% radioactive label											
		Benign breast						Normal breast					
	Patient A	Patient B	Patient C	Patient D	Patient E	Patient A	Patient B	Patient D	Patient F	Patient G	Patient H		
Testosterone	22.96	2.21	73.38	87.43	77.30	95.50	73.20	95.20	79.90	73.60	98.50		
5 α -DHT	20.25	48.47	9.55	0.25	0.24	1.72	8.02	0.19	0.50	0.89	0.06		
5 α -A'-diol	9.16	8.77	1.74	0.49	0.30	0.14	0.51	0	0.30	0.13	0		
4-dione	—	—	14.55	—	15.00	—	—	—	—	—	—		

5 α -A'-diol = 5 α -androstanediol; 4-dione = 4-androstenedione.

* Fraction lost.

RESULTS

The results are presented in Tables 1 and 2. It may be seen that significant amounts of both the ^3H -DHA and ^{14}C -testosterone precursors were metabolized by normal and benign female breast tissue. Testosterone was a metabolite of DHA and 5α -dihydrotestosterone (DHT) and 5α -androstanediol metabolites of testosterone. In contrast, only very small amounts of the steroid substrates were metabolized by the gynaecomastic breast tissue. In no incubation was testosterone produced from DHA and only 5α -DHT and 4-androstenedione but not 5α -androstanediol were metabolites of testosterone.

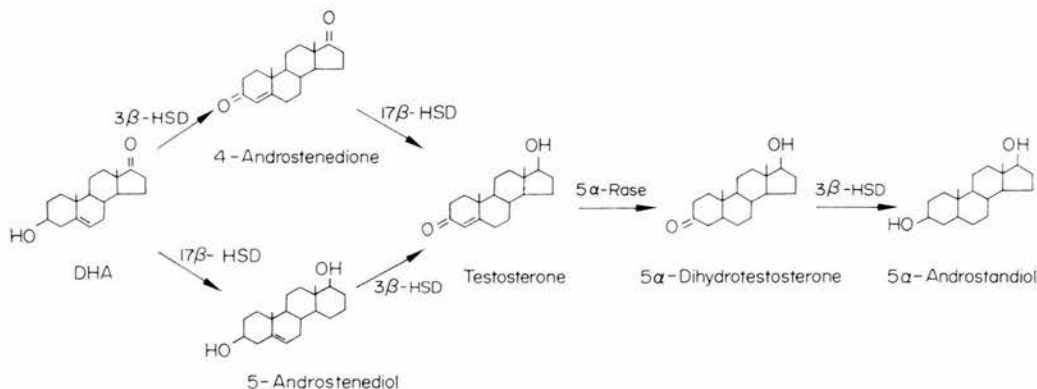


FIG. 3. 3β -HSD = 3β -hydroxysteroid dehydrogenase; 17β -HSD = 17β -hydroxysteroid dehydrogenase; 5α -Rase = 5α -reductase.

DISCUSSION

The inability to detect in gynaecomastic tissue the conversion of DHA to testosterone or testosterone to 5α -androstanediol is interesting since other steroid interconversions such as the transformation of testosterone to 5α -DHT and 4-androstenedione were clearly demonstrable. The pathways of these conversions in other endocrine organs are presented in Fig. 3. It may be seen that the conversion of DHA to testosterone requires both a 3β -hydroxysteroid dehydrogenase (3β -HSD) and a 17β -hydroxysteroid dehydrogenase (17β -HSD) whilst the conversion of testosterone to 5α -androstanediol requires both a 5α -reductase and a 3β -HSD. Since the gynaecomastic tissue was able to transform testosterone to 5α -DHT and 4-androstenedione, however, such tissue must possess both the 5α -reductase and 17β -HSD activities. It would seem, therefore, that gynaecomastic breast tissue lacks 3β -HSD activity.

This activity was, however, clearly demonstrable in the female breast tissue investigated. It is unlikely that this difference is sex linked as 3β -HSD activity has been shown to be present in both normal male and female tissue such as skin (Baillie *et al.*, 1965; Hodgins, 1971) and thyroid tissue (W. R. Miller, unpublished results). Indeed 3β -HSD activity is almost ubiquitous in mammalian tissue (Dorfman & Ungar, 1965).

The absence of 3β -HSD activity in gynaecomastic tissue could be caused by either a genetic defect or by complete inhibition/repression of the enzymatic activity which could be demonstrated under the appropriate conditions. The first possibility is interesting as certain familial instances of gynaecomastia do occur (Hall, 1958; Peters *et al.*, 1955) although there was no

evidence for such in the patients from the present study. It might be expected in such cases that the defect may also be present in other tissues normally possessing the enzyme activity. Bercovici & Mauvais-Jarvis (1972) have shown, however, that the 'cutaneous' metabolism of testosterone to androstanediol is normal in patients with gynaecomastia associated with hyperthyroidism.

The activity of 3β -HSD may be hormonally controlled. In the testes, for example, the activity may be influenced by both steroid and pituitary hormones (Hafiez *et al.*, 1971). Thus it is possible that the 3β -HSD activity may be inhibited or repressed in gynaecomastic tissue, particularly in patients with conditions producing a marked circulating hormone imbalance such as patients with Klinefelter's syndrome, testicular tumours and those receiving oestrogen therapy.

A question as yet unanswered is whether this *in vitro* absence of activity, if reflected *in vivo*, would alter the cellular hormonal environment of the breast sufficiently to induce gynaecomastic growth. By reducing the production of testosterone, the absence of the activity *in vivo* could lead to a local tissue imbalance in the androgen : oestrogen ratio. This would be in keeping with the observation that many cases of gynaecomastia are associated with states of hyperoestrogenaemia or hypoandrogenaemia (Bercovici & Mauvais-Jarvis, 1972; Korenman *et al.*, 1969; Lazarev *et al.*, 1969).

The role of 3β -HSD in human breast tissue requires further clarification. It is recognized that female breast tissue is not the ideal control tissue for gynaecomastic breast but comparative studies are handicapped by the difficulty of acquiring suitable non-gynaecomastic breast tissue. It is hoped to perform further studies should such tissue become available.

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ŒSTRADIOL SYNTHESIS BY A HUMAN BREAST CARCINOMA

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Summary Unequivocal evidence is presented for the synthesis of œstradiol-17 β from an androgen precursor by a human breast carcinoma.

Introduction

EVIDENCE that human breast carcinoma can synthesise œstrogen is unconvincing. The report by Adams and Wong¹ that microsomal preparations of breast carcinoma could convert testosterone to œstriol has not been confirmed; contamination with androgen impurities may explain their findings.² The reported transformations of both dehydroepiandrosterone and Δ^4 -androstenedione to œstrone³ also have not been confirmed; in any event, they were extremely small. Using a dual isotope technique, we have been unable to demonstrate the production of either œstrone or œstradiol-17 β from both dehydroepiandrosterone and testosterone in three different breast carcinomas.⁴ However, we now report the synthesis of œstradiol-17 β in significant amounts by a tumour incubated with the single precursor, 7 α^3 H-testosterone.

Methods

The patient, a 55-year-old woman, was referred in July, 1973, with advanced cancer of the breast with extensive local disease and pulmonary and skeletal metastases. She was 5 years post-menopausal and, following biopsy of the tumour, which was histologically described by Dr A. H. Wylie as an anaplastic pleomorphic carcinoma of the breast with considerable mitotic activity (fig. 1), she was treated with tamoxifen ('Novaldex', I.C.I.) 10 mg. twice daily by mouth. She was readmitted to hospital for assessment 6 weeks after starting the drug and at that time was adjudged to have shown no response to the treatment—this on the basis of advancement of the pulmonary lesions, and the development of neurological symptoms and signs suggesting cerebral metastases. Her disease progressed and she died in October, 1973.

Tumour for incubation was obtained at the time of biopsy before the patient had started therapy.

The tissue was finely sliced and divided into three portions of 1 g. for incubation in triplicate. The slices were suspended in 10 ml. Krebs/Ringer phosphate buffer containing an N.A.D.P.H. generating system and 50 μ Ci $7\alpha^3$ H-testosterone. One incubation was used without further addition as a control system. Either unlabelled α estradiol-17 β (1.5 μ g. per ml.) or rat prolactin (50 μ g. per ml.) was added to the other incubations to determine the effects of these hormones. All systems were then

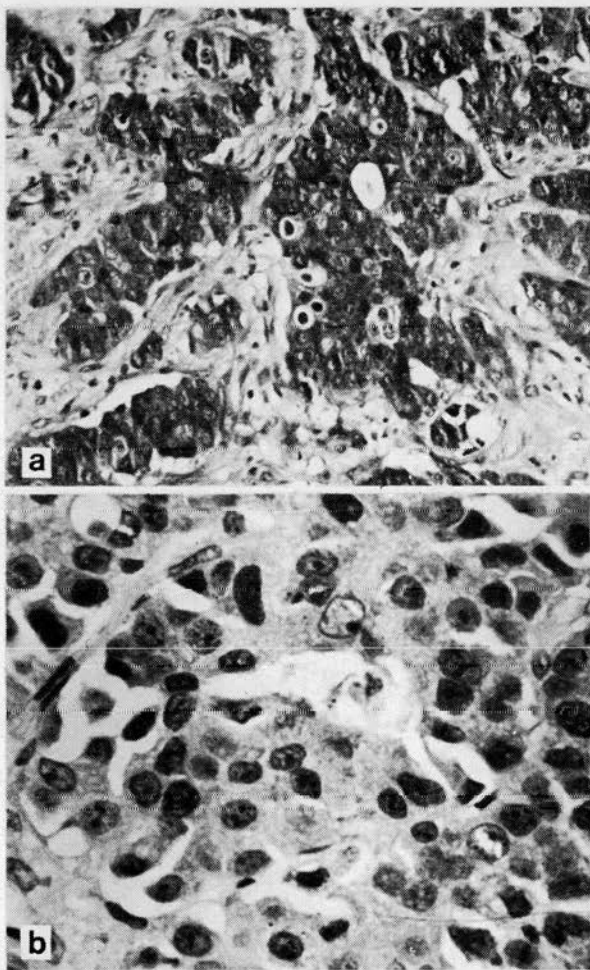


Fig. 1—Photomicrographs of representative sections of tumour studied.

a, $\times 64$; b, $\times 160$. Hæmatoxylin and eosin.

incubated by shaking for 2 hours at 37°C in an atmosphere of oxygen. The various conversions were determined by measuring the percentage incorporation of tritium into the metabolites. Details of the methods of steroid purification, characterisation, and measurement have been described elsewhere.⁵

The determination of high affinity-binding activity for oestradiol-17 β was also assayed by the method of Feherty et al.⁶

Results

The percentage radioactivity found in those metabolites which were investigated is shown in table I. The evidence for the production of oestradiol-17 β is presented in table II. This is based upon the consistency of specific radioactivity during the formation of chemical derivatives. Although there are differences between the three incubations, it can be

TABLE I—METABOLISM OF 7 α^3 H-TESTOSTERONE

—	Control	+ oestradiol	+ prolactin
Testosterone	82.97	81.15	83.97
5 α -dihydrotestosterone ..	1.67	1.19	1.41
5 α -androstanediol	0.85	0.74	0.63
Δ 4-androstenedione	4.71	3.42	4.18
Oestradiol-17 β	0.37	0.21	0.32

TABLE II—EVIDENCE FOR THE IDENTIFICATION OF OESTRADIOL-17 β

—	—	d.p.m.	Cold (nM)	Sp. ac. d.p.m./ nM	% con- version
Control	Oestradiol	230,400	1000	230	0.37
	OE ₂ diacetate	166,400	740	225	
	OE ₂ diacetate	85,450	405	211	
	hydrolysed				
	OE ₂ methyl ether	51,600	225	229	
+ OE ₂ (1.5 μ g./ml.)	Oestradiol	119,400	920	130	0.21
	OE ₂ diacetate	95,650	755	127	
	OE ₂ diacetate	49,700	390	127	
	hydrolysed				
	OE ₂ methyl ether	29,750	245	121	
+ prolactin (50 μ g./ml.)	Oestradiol	166,550	875	190	0.32
	OE ₂ diacetate	125,250	665	188	
	OE ₂ diacetate	73,250	375	195	
	hydrolysed				
	OE ₂ methyl ether	48,050	235	204	

OE₂ = Oestradiol.

seen that oestradiol-17 β was a metabolite of testosterone in each. Δ 4-androstenedione was the major metabolite identified and, as reported previously,⁴ 5 α reduced products, such as 5 α -dihydrotestosterone and 5 α -androstenediol, were formed in significant amounts.

Oestrogen-receptor activity was not demonstrated.

Discussion

These results are the first demonstration that human breast cancer can convert a C19 steroid (testosterone) into oestradiol-17 β . Taking into account previous studies, one can now construct the metabolic pathways for steroid metabolism by human breast cancer indicated in fig. 2.

The formation of oestradiol-17 β from a C19 steroid has important implications in understanding the mechanism of hormone dependence of human breast cancer. Firstly, it offers, within a unified concept of oestradiol dependence, an explanation for the beneficial effect of oophorectomy in the premenopausal woman and of adrenalectomy and hypophysectomy in the postmenopausal woman. The benefit from oophorectomy has long been accepted as being due to reduction in the levels of circulating oestrogens, which in the premenopausal woman vary between 35 and 200 pg. per ml. during each menstrual cycle.⁷ However, one cannot explain the effect of adrenalectomy in the postmenopausal patient on a similar basis. Not only are circulating levels of oestrogen low,⁷ but the human adrenal cortex secretes small and relatively insignificant amounts of oestrogen.⁸

On the other hand, the adrenal cortex secretes large amounts of C19 steroids, particularly dehydroepiandrosterone sulphate, circulating levels of which in postmenopausal women may exceed 50 μ g. per 100 ml. plasma.⁹ Conversion of dehydroepiandrosterone and its sulphate to testosterone by breast-cancer tissue has been previously demonstrated.^{3,4} That of testosterone to oestradiol-17 β in the tumour under consideration further indicates that this metabolic sequence can result in the local formation of active oestrogen. Reduction of dehydroepiandrosterone-sulphate secretion following adrenalectomy may therefore effect a reduction in the concentrations of oestradiol-17 β at tumour level.

A similar mechanism could explain the remission

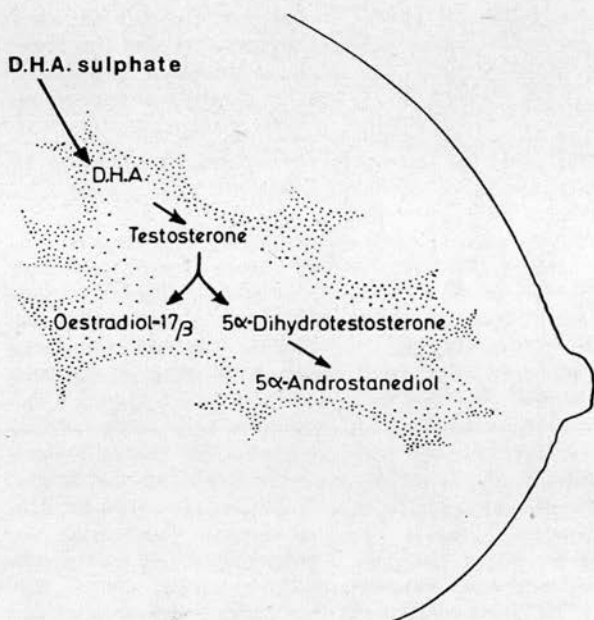


Fig. 2—Steroid biosynthetic pathways demonstrated in human breast cancer.

D.H.A. = dehydroepiandrosterone.

of breast cancer which may occur following hypophysectomy and pituitary-stalk section, and which cannot be explained by deprivation of prolactin. Following pituitary-stalk section, prolactin levels are elevated, yet remission of tumour growth has been described.¹⁰ On the other hand, both procedures suppress the secretion of corticotrophin, which is known to control the production of dehydroepiandrosterone sulphate by the adrenal cortex.¹¹

Secondly, the conversion of a C19 steroid to an active oestrogen at tumour level may also offer an explanation for the observation that tumours may be sensitive *in vitro* to both androgens and oestrogens, or even to androgens alone.¹² The presence or absence of oestrogen-receptor activity and possibly androgen-receptor activity may also contribute to these differences.

The tumour under study not only converted testosterone to oestradiol-17 β by aromatisation of its A ring, but also, by 5 α reduction of the same ring, produced the biologically active androgens—5 α -

dihydrotestosterone and 5α -androstanediol. In the rat, 5α -dihydrotestosterone has been shown to inhibit the growth of mammary tumours,¹³ whereas oestrogen stimulates them.¹⁴ It is thus possible that the relative production of 5α -dihydrotestosterone and oestradiol- 17β from the same precursor at tumour level may be of importance in determining the influence of hormones on tumour growth.

The control of these metabolic pathways is uncertain. We have recently shown that 5α -reductase activity in dimethylbenzanthracene-induced rat mammary tumours is enhanced by the induction of a state of hyperprolactinaemia.¹⁵ Studies are now in progress to determine whether similar controlling mechanisms can be identified in human tumours. Although the three incubations which were performed in this study included two in which oestradiol or prolactin were added, any effect of these hormones cannot be discussed on the basis of a single tumour. It is notable, however, that in all three separate incubations the synthesis of biologically potent oestrogen from testosterone was demonstrated.

No firm conclusions can be drawn regarding the hormone dependence of the tumour studied. Although tamoxifen had no effect on the progressive growth of the tumour, it was at a very advanced stage when the patient first was seen and the likelihood of response was not great. Nor can any firm conclusions be reached about the significance of failure to detect oestrogen-receptor activity. The method used identifies only unoccupied binding sites; in the presence of significant endogenous oestrogen production these may well be few. However, it is tempting to postulate that for this tumour a more appropriate treatment might have been adrenalectomy.

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Short CommunicationOESTRADIOL SYNTHESIS FROM C19 STEROIDS BY
HUMAN BREAST CANCERS

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THE REGRESSION of advanced breast cancer which can follow ovarian ablation is believed to be due to reduction in the levels of circulating oestrogen. The benefit which may follow adrenalectomy in oophorectomized and in post-menopausal women cannot be explained on a similar premise. In post-menopausal women plasma oestrogens are already low (England *et al.*, 1974) and the adrenal cortex secretes only trace amounts of oestrogen. The main sex hormones secreted by the adrenal cortex are C19 steroids (Cameron *et al.*, 1969) which we and others have shown to be metabolized by breast tumours (Adams and Wong, 1968; Jones *et al.*, 1970; Jenkins and Ash 1972; Miller *et al.*, 1973). Recently we gave unequivocal evidence that the C19 steroid, testosterone could be utilized by a human breast cancer to synthesize oestradiol-17 β (Miller and Forrest, 1974). The aim of this study was to determine whether this effect was reproducible in other tumours.

MATERIALS AND METHODS

Patients.—Thirteen patients with proved cancer of the breast were studied. Eight subjects were at least 5 years postmenopausal, a further 2 were less than 5 years postmenopausal and 2 more were experiencing regular menstrual periods at the time of investigation. The remaining patient had been oophorectomized 2 years before the study.

Tumour processing and incubation.—Following excision, the tumours (11 primary

and 2 secondary recurrences from the chest wall) were put on ice in the operating theatre. Sufficient tissue was removed by a pathologist for histological diagnosis and the remainder of the tumour was finely sliced and incubated for 2 h at 37°C in Krebs Ringer phosphate buffer pH 7.4 (10 ml/g tissue), containing an NADPH generating system and 45 μ Ci 7 α -³H testosterone. The metabolism of testosterone was then determined by measuring the percentage of incorporation of ³H into the various purified metabolites. Details of the methodology used for steroid purification, characterization by chemical derivatives and measurement have been described previously (Miller, Forrest and Hamilton, 1974). Identification of oestradiol-17 β fractions was based on the following criteria: (a) the fractions on acetylation and methylation formed compounds which, on thin layer chromatography, moved with the same mobility as authentic oestradiol diacetate and oestradiol-3-methyl ether respectively; (b) consistent specific radioactivity was maintained throughout derivative formation.

RESULTS

The percentage radioactivity found in the various metabolites investigated is shown in Table I.

All tumours metabolized testosterone but with considerable variation (17–54%). The presence of 5 α reductase activity was demonstrated in all tumours and both 5 α dihydrotestosterone and 5 α androstanediol were identified as metabolites. The level of production of 5 α dihydrotestosterone invariably exceeded that of 5 α andros-

TABLE I.—*Metabolism of $7\alpha^3\text{H}$ Testosterone by Human Breast Carcinomata*

	% Metabolism	% Conversion to			
		5 α Dihydrotestosterone	5 α Androstenediol	Δ 4 Androstenedione	Oestradiol-17 β
EC	17.03	1.67	0.85	4.71	0.37
JC	39.35	1.93	0.69	6.76	0.22
E.Cr	53.79	0.65	0.13	38.49	0.07
A.R.	24.55	2.79	1.18	7.83	0.06
ES	24.48	0.37	0.09	0.39	0.05
CMcD	19.92	0.41	0.09	2.03	0.04
CR	28.94	0.72	0.16	6.90	neg?
GM	26.93	0.58	0.21	3.84	neg?
G.A.	33.36	2.61	1.18	2.91	neg?
MMcN	29.59	0.91	0.38	4.83	neg?
J.M.	27.66	3.04	1.50	0.60	0
MR	27.66	3.04	1.50	0.60	0
JR	27.10	0.44	0.15	0.65	0

neg? = low inconsistent specific radioactivity.

TABLE II.—*Evidence for the Identification of Oestradiol 17 β*

	Derivative	Specific activity d/min/nmol	% Conversion
E.C.	Oestradiol free	211	0.37
	Oestradiol diacetate	225	
	Oestradiol methyl ether	229	
J.C.	Oestradiol free	126	0.22
	Oestradiol diacetate	118	
	Oestradiol methyl ether	121	
E.Cr.	Oestradiol free	44.7	0.08
	Oestradiol diacetate	45.5	
	Oestradiol methyl ether	41.6	
A.R.	Oestradiol free	35.3	0.06
	Oestradiol diacetate	33.8	
	Oestradiol methyl ether	34.9	
E.S.	Oestradiol free	31.7	0.05
	Oestradiol diacetate	31.8	
	Oestradiol methyl ether	29.2	
CMcD	Oestradiol free	17.8	0.04
	Oestradiol diacetate	19.1	
	Oestradiol methyl ether	18.2	

tanediol. All tumours converted testosterone to Δ 4 androstenedione and, in most, this steroid represented the single greatest metabolite identified.

Unequivocal evidence (Table II) for the synthesis of oestradiol-17 β was found in 6 tumours, in 2 of which the amounts were substantial. In 7 tumours oestradiol-17 β was not identified. Although in 4 of these small amounts of radioactive label were incorporated in the crude oestradiol fraction, consistent specific radioactivity was not obtained in the derivatives.

COMMENT

These findings confirm that all human breast cancers can metabolize C19 steroids.

Furthermore, all tumours studied had 5 α reductase activity and were able to convert testosterone into its 2 active 5 α reduction products, 5 α dihydrotestosterone and 5 α androstenediol.

In contradistinction, not all tumours could synthesize oestradiol-17 β and we conclude that the possession of the aromatizing system is specific to certain types of tumour. To date, we have not uncovered any particular difference between those tumours which have oestradiol synthesizing capacity and those which do not.

Since biologically approximately half of all human breast cancers do show some degree of hormone dependence and one-third markedly so, it is tempting to believe

that the possession of aromatizing enzymes may be of importance in this regard. In this event, the tumours which were capable of transforming C19 steroid into oestrogen could be those which are dependent on the adrenal cortical source of C19 steroids.

We have already suggested that the beneficial effects of adrenalectomy and hypophysectomy could be due to reduction of circulating C19 precursor steroids such as DHA sulphate (Miller and Forrest, 1974). The results we now report are further evidence of such a possibility.

Studies are now in progress to determine the relationship of possession of this synthetic pathway to oestrogen receptor activity and to the clinical response to adrenalectomy and hypophysectomy.

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Factors affecting testosterone metabolism by human breast tissues

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The *in vitro* metabolism of $7\alpha^3\text{H}$ -testosterone by 36 human breast carcinomas and 4 specimens of 'normal' breast has been studied. All metabolized testosterone to 5α -dihydrotestosterone, 5α -androstenediol and $\Delta 4$ -androstenedione. Evidence for synthesis of oestradiol- 17β was obtained in 17 of the 36 carcinomas but in no specimen of 'normal' breast. 5α -reduction of testosterone was significantly higher in tumours from premenopausal patients and those with a high proportion of parenchyma. Oestradiol production was also highest in tumours with the least 'stromal reaction'.

Introduction

There is now abundant evidence that human breast cancer possesses paraendocrine properties in its potential to synthesize steroid hormones. The C₁₉ steroid, dehydroepiandrosterone sulphate, which circulates in large amounts even in postmenopausal women (Wang *et al.*, 1976), has been shown to be metabolized to testosterone (Jones, Cameron, Griffiths, Gleave & Forrest, 1970; Miller, McDonald, Forrest & Shivas, 1973). Furthermore, testosterone itself may be transformed into the active androgen 5α -dihydrotestosterone (Jones *et al.*, 1970; Miller *et al.*, 1973; Adams & Li, 1975; Jenkins & Ash, 1972) and, although initial reports of conversion to oestrogen (Adams & Wong, 1968) may be explained on the basis of androgen impurities (Dao, 1969), we have presented unequivocal evidence for the metabolism of testosterone to oestradiol (Miller & Forrest, 1974) and others have reported the synthesis of small amounts of oestrone from $\Delta 4$ -androstenedione (Jones *et al.*, 1970). Tumour oestradiol production has since been confirmed by others (de Thibault de Boesinghe, Lacroix, Eechante & Leusen, 1974; Abul-Hajj, 1975) but it appears that only a proportion of breast cancers possess the potential (Abul-Hajj, 1975; Miller & Forrest, 1976). We now consider some factors which might influence the aromatization of testosterone and also its 5α -reduction to active androgens.

Methods

Patients

Tumour was obtained from 36 women, 24 with primary and 12 with metastatic or recurrent breast cancer. In 4 patients, 'normal' tissue was also taken from an area of the breast as remote as possible from the primary tumour. Seven patients were premenopausal (experiencing regular menstrual periods), 26 were postmenopausal (at least 5 years since their last regular menstrual period) and 3 menopausal (0 to 5 years from their last regular menstrual period).

Tissues

Following excision tissues were immediately put on ice, finely sliced and incubated for 2 h at 37°C in Krebs Ringer phosphate buffer pH 7.4 (15 ml per g tissue), containing an NADPH-generating system and 7 α H³-testosterone (45 μ Ci per g tissue). The reaction was stopped by adding methanol to 80% v/v and the incubations were stored at -10°C.

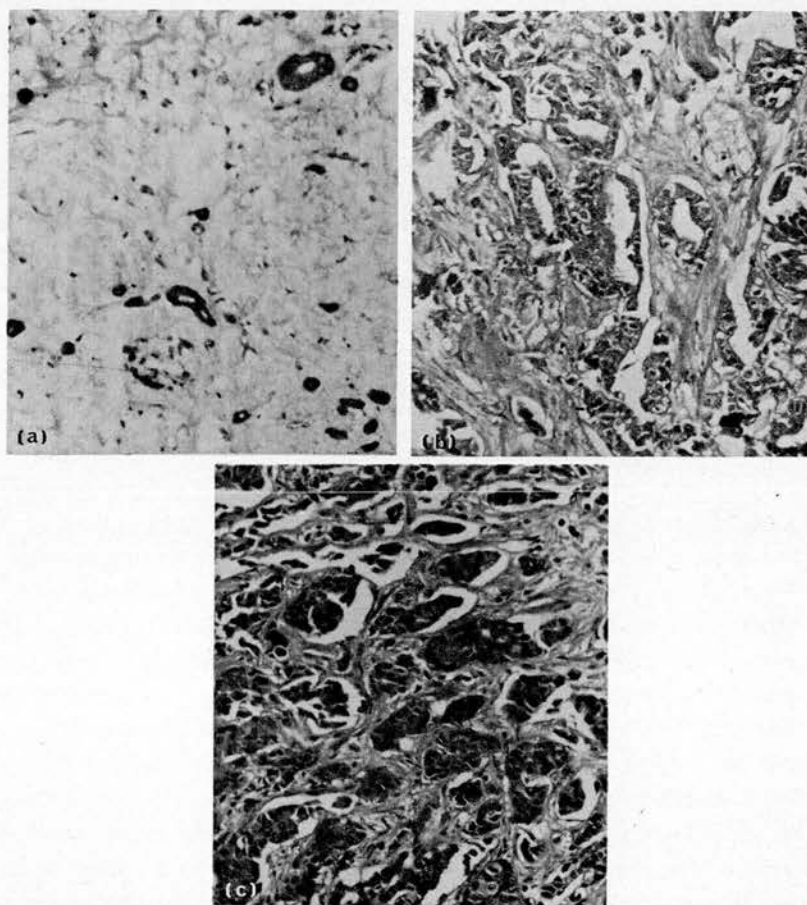


Figure 1. Examples of grading of tumour differentiation. (a) A well differentiated adenocarcinoma. Dispersed neoplastic elements consist of well formed tubules or acini. HE. $\times 125$. (b) A moderately differentiated carcinoma showing some glandular elements of variable atypism, associated with more anaplastic growth. HE. $\times 125$. (c) An undifferentiated (or anaplastic) carcinoma in which irregular clumps and trabeculae of tumour cells are devoid of architecture. HE. $\times 125$.

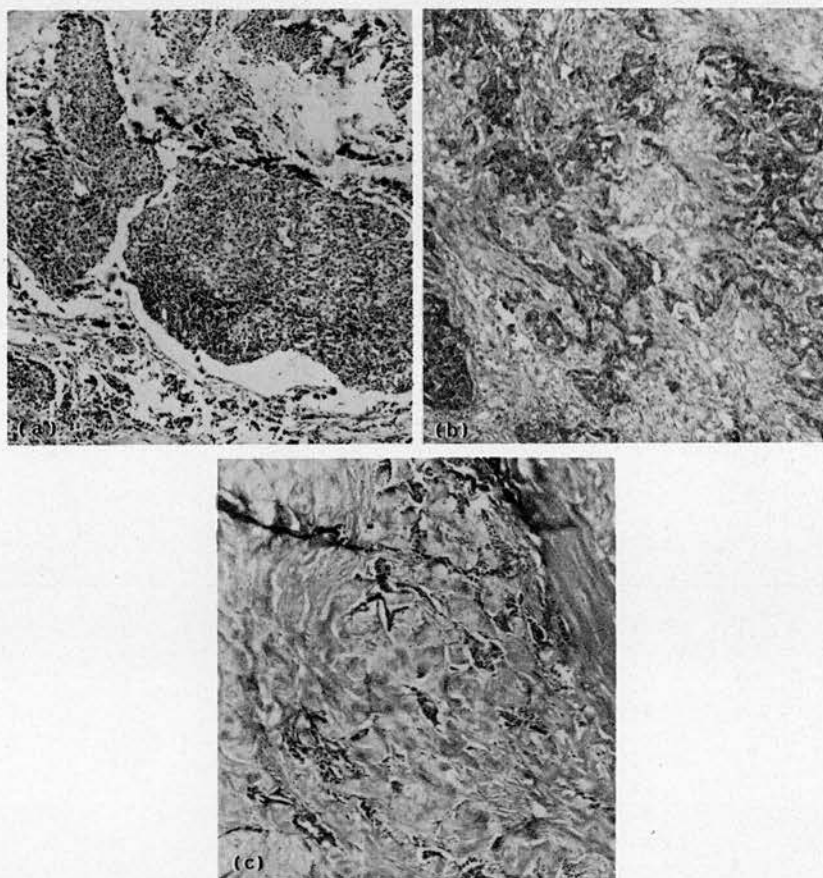


Figure 2. Example of grading of tumour 'stromal reaction'. (a) Low 'stromal reaction'—a carcinoma of high cellularity, having only a small component of connective tissue. HE. $\times 50$. (b) Moderate 'stromal reaction'—a carcinoma of moderate cellularity, in which a substantial component of connective tissue is associated with proportionately fewer tumour cells. HE. $\times 50$. (c) Marked 'stromal reaction'—a carcinoma of low cellularity and correspondingly large element of connective tissue—a typical 'scirrhous' carcinoma of breast. HE. $\times 50$.

Before extraction of the metabolites, 500 μg of non-radioactive carriers of those steroids to be characterized were added to monitor losses. The metabolites were then extracted, separated into individual steroids and purified by thin-layer chromatography by the methods previously described (Miller, Forrest & Hamilton, 1974). The percentage incorporation of ^3H into the various purified metabolites was measured and the metabolites characterized by chemical derivative formation. The criteria for identification of oestradiol- 17β have been previously described (Miller & Forrest, 1976). 5α -reduction was estimated by combining the percentage production of 5α -dihydrotestosterone (5α -DHT) and 5α -androstenediol for each tumour.

Histology

Sufficient material was available for a valid assessment of the histopathology of the tumour in 31 cases. The pathologist (A.A.S.), who did not previously know the steroidogenic potential of the tumours, graded them according to their differentiation

TABLE 1. Metabolism of $7\alpha^3\text{H}$ -testosterone by normal human breast

Patient	% metabolism	5α -DHT	% conversion to		Oestradiol- 17β
			5α -androstane- 3α -diol	Δ^4 -androstenedione	
2	35.30 (39.35)	0.06 (1.93)	0.05 (0.69)	2.30 (6.76)	0 (0.22)
4	24.50 (21.50)	0.11 (0.57)	0.11 (0.25)	0.20 (1.46)	0 (0.17)
14	17.44 (24.48)	0.12 (0.37)	0.12 (0.09)	0.87 (0.39)	0 (0.05)
15	25.50 (18.92)	4.70 (0.41)	0.78 (0.09)	2.26 (2.03)	0 (0.04)

(Figures in parenthesis represent % conversions in malignant tissue associated with same patient.)

TABLE 2. Metabolism of $7\alpha^3\text{H}$ -testosterone by human breast carcinomata

Patient	% metabolism	5α -DHT	% conversion to		Oestradiol
			5α -androstane- 3α -diol	Δ^4 -androstenedione	
1	17.03	1.67	0.85	4.71	0.37
2	39.35	1.93	0.69	6.76	0.22
3	10.70	1.59	1.40	1.63	0.21
4	20.50	0.57	0.25	1.46	0.17
5	18.65	0.87	0.10	5.19	0.12
6	16.20	0.79	0.30	0.30	0.10
7	18.35	3.85	1.31	3.54	0.08
8	18.26	0.49	0.46	2.30	0.08
9	53.79	0.65	0.13	38.49	0.07
10	24.55	2.79	1.18	7.83	0.06
11	24.92	2.73	0.71	1.43	0.06
12	15.91	0.98	0.51	2.87	0.06
13	18.29	1.70	0.49	0.54	0.06
14	24.48	0.37	0.08	0.39	0.05
15	18.92	0.41	0.09	2.03	0.04
16	23.24	1.70	0.66	1.20	0.04
17	20.79	0.55	0.43	0.49	0.03
18	24.32	3.77	1.67	0.58	0
19	21.80	1.29	0.97	0.58	0
20	26.83	0.58	0.21	3.48	0
21	26.62	1.05	0.94	6.38	0
22	28.94	0.72	0.16	6.90	0
23	33.36	2.61	1.18	2.19	0
24	29.59	0.91	0.38	4.83	0
25	27.66	3.04	1.50	0.60	0
26	27.10	0.44	0.15	0.65	0
27	11.12	0.94	0.74	5.14	0
28	17.42	1.28	0.37	0.93	0
29	40.41	3.66	0.85	1.73	0
30	10.05	1.08	0.35	0.37	0
31	11.04	2.55	1.03	1.47	0
32	39.02	0.60	0.18	0.79	0
33	23.34	1.07	0.32	9.42	0
34	20.05	1.42	0.36	0.49	0
35	18.20	1.13	0.56	0.38	0
36	16.86	1.76	1.35	3.31	0

(by an assessment of the extent of glandular formation) and 'stromal reaction' (ratio of tumour connective tissue to parenchyma). Typical examples of grading are shown in Figures 1 and 2.

Paraffin sections confirmed that 'normal' tissue was free of malignant or recognizable premalignant change.

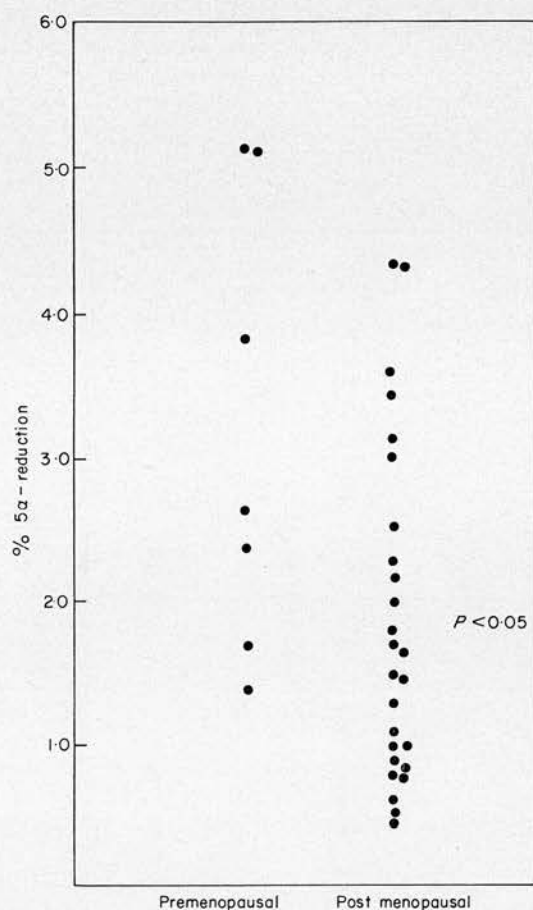


Figure 3. Comparison of %5 α -reduction in tumours from premenopausal and postmenopausal patients. Significant difference between the groups by Wilcoxon Rank test ($P < 0.05$).

Results

All 4 incubations of normal breast metabolized testosterone to 5 α -DHT, 5 α -androstenediol and Δ 4-androstenedione. In none was oestradiol-17 β identified. In Table 1 the associated results of incubations of the 4 carcinomas *from the same breasts* are also given. It is notable that all *tumours* synthesized oestradiol-17 β .

All 36 tumours converted testosterone to 5 α -DHT, 5 α -androstenediol and Δ 4-androstenedione (Table 2). In most, the major metabolite identified was Δ 4-androstenedione and in all the transformation to 5 α -DHT exceeded that to 5 α -androstenediol. Oestradiol synthesis was identified in 17 tumours and reached or exceeded 0.1% conversion in 6.

Menopausal status

As is shown in Figure 3 the 5 α -reduction of testosterone was significantly higher in premenopausal patients as compared with those from postmenopausal subjects. In contrast, conversion to Δ 4-androstenedione and oestradiol-17 β was not influenced by menopausal status. Furthermore, potential for tumour oestrogen synthesis did not differ between menopausal groups (Table 3).

TABLE 3. The effect of menopausal status upon oestradiol synthesis by human breast carcinomas

	No. of tumours	No. with oestradiol synthesis	Range of conversion
Premenopausal	7	3	0-0.22%
Menopausal	3	2	0-0.06%
Postmenopausal	26	12	0-0.37%

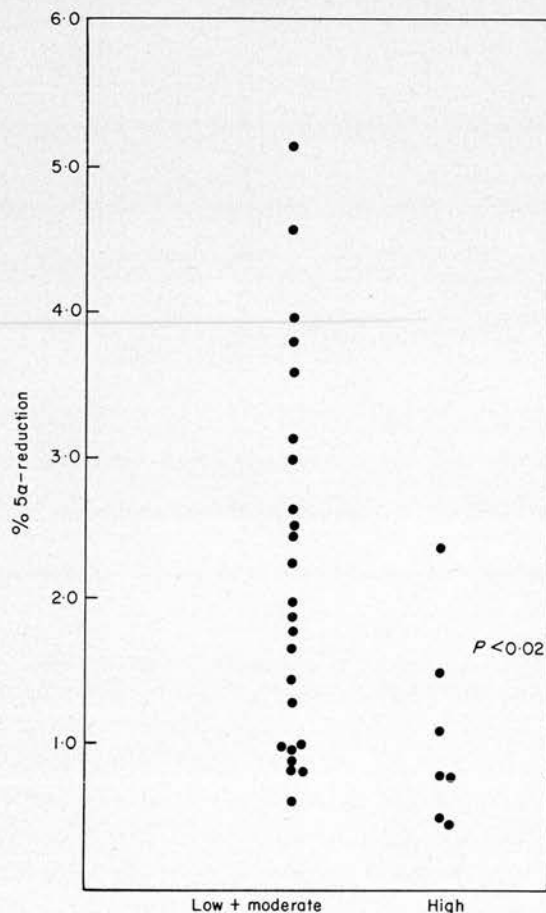


Figure 4. Comparison of %5 α -reduction between tumours of low and moderate 'stromal reaction' and those with high 'stromal reaction'. Significant difference between the groups by Wilcoxon Rank test ($P < 0.02$).

Histopathology

The majority of the 31 tumours available for histological examination were poorly differentiated, only a single tumour being characterized as well-differentiated and 5 carcinomas as moderately differentiated. No relationship was detected between degree of tumour differentiation and any aspect of the metabolism of testosterone.

Assessment of the ratio of stroma to tumour parenchyma showed 7 tumours with low, 17 with moderate and 7 with marked 'stromal reaction'. There was no relationship between the amount of 'stromal reaction' and the level of testosterone metabolized

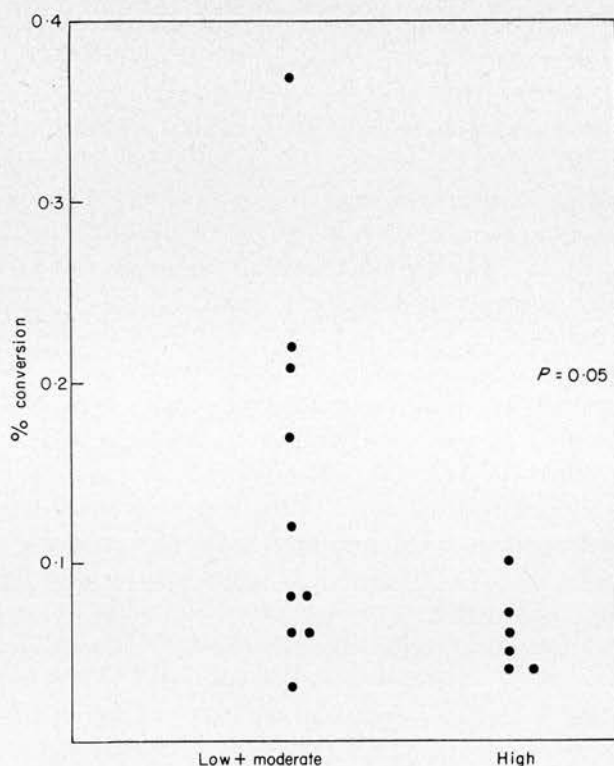


Figure 5. Comparison of % conversion to oestradiol-17 β between tumours of low and moderate 'stromal reaction' and those with high 'stromal reaction'. Significant difference between the groups by Wilcoxon Rank test ($P=0.05$).

or its conversion to Δ^4 -androstenedione. However, the 5α -reduction of testosterone in tumours with low and moderate amounts of stroma i.e. those with the greatest proportion of parenchyma, was significantly higher than that in tumours with marked stromal reaction (Figure 4). Similarly, the level of oestradiol synthesis was significantly higher in tumours with lowest stroma and greatest parenchyma (Figure 5). However, there was no correlation between degree of 'stromal reaction' and the absolute potential for oestradiol synthesis.

Discussion

These results confirm our previous reports that all human breast cancers and 'normal' breast can convert testosterone to 5α -DHT and 5α -androstane-3 α ,20 β -diol (Miller *et al.*, 1973; Miller & Forrest, 1974). Tumour 5α -reduction tends to be higher in premenopausal women. Whether this is an inherent difference in tumours from premenopausal women or results from the higher level of circulating hormones in premenopausal subjects is not known. Although 5α -reductase is influenced by the endocrine environment in rat mammary carcinomas, oophorectomy increases the activity and administration of oestrogen decreases 5α -reduction (Miller, 1976). Furthermore, the difference cannot be explained solely on the basis of premenopausal tumours being the most cellular. Comparison of Figures 3 and 4 show that amongst

postmenopausal tumours with low 5 α -reduction are tumours with relatively high cellularity. Contrary to the findings of Jenkins & Ash, who studied a very small number of tumours, there was no relationship between the degree of differentiation of each tumour and its 5 α -reductase. However, in the present study, the number of differentiated tumours is still small. The production of 5 α -reduced metabolites was, however, low in tumours with marked 'stromal reaction'. This suggests that, although fibroblasts possess 5 α -reductase activity (Mulay, Finkelberg, Pensky & Solomon, 1972), connective tissue elements of human breast carcinoma show comparatively low 5 α -reduction and the activity is primarily associated with other tumour cells.

Conversion of testosterone to oestradiol was identified in only half the tumours studied. Aromatization was not observed in 'normal' breast, even when tumour within it had this enzymic activity. This might indicate a change with the development of neoplasia or it could equally be a measure of the low cellular content of 'normal' breast tissue. Like others (de Thibault de Boesinghe *et al.*, 1974), we failed to find any correlation between the menstrual status of the patient or the histopathology of the tumour and its capacity to synthesize oestrogen. Nevertheless, the amounts of oestradiol produced were generally less in tumours with marked 'stromal reaction'. The quantitatively higher synthesis of oestrogen in tumours with a high proportion of parenchyma and the inability to detect synthesis in normal breast suggest that aromatizing activity, when present, is primarily associated with tumour parenchyma.

In many tumours, a high percentage of the testosterone metabolized remained unaccounted for. It was our experience, however, that most of the uncharacterized radioactivity was associated with highly polar compounds. This would be in agreement with the results of others who have found polyhydroxylated metabolites in incubations of breast tumours (Dao, Varda & Morreal, 1972).

The findings in this study suggest that whilst the interconversion of C₁₉ steroids is commonly associated with the breast and its tumours, the possession of aromatizing ability is a more selective property. It still remains to determine what factors influence whether an individual tumour synthesizes oestrogen or not and whether the capacity for aromatization is associated with the hormone dependency of the tumour.

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Inhibition of Aromatization by Aminoglutethimide in Breast Cancers — Clinical Relevance

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Aminoglutethimide (Orimetan®) therapy in post-menopausal patients with advanced breast cancer gives results comparable with those of surgical adrenalectomy; remissions occur in about 30% of women (Wells *et al.*, 1982). This "medical adrenalectomy", so called because the drug markedly reduces adrenal production of steroid hormones (Wells *et al.*, 1978), is achieved in part by blocking conversion of cholesterol to pregnenolone, the initial step in the biosynthetic pathway for steroid hormones. In addition, however, work originally performed with preparations from placenta (Thompson and Siiteri, 1974) has shown that aminoglutethimide specifically inhibits the aromatization reaction which converts androgens into oestrogens. This is relevant to the treatment of breast cancer because tumours responding to aminoglutethimide usually contain cytoplasmic receptors for oestrogen (Santen and Wells, 1980).

However, the adrenal cortex produces little oestrogen (Baird *et al.*, 1969), and in post-menopausal women most oestrogen is formed by extra-adrenal aromatization of androgen precursors (Grodin *et al.*, 1973) which are primarily derived from the adrenal. Sites of peripheral aromatization include adipose tissue, muscle and skin (Longcope *et al.*, 1978). In the patient with breast cancer, there is potentially another important site—the tumour itself (Miller and Forrest, 1974, 1976).

In vitro studies of steroid metabolism by human breast cancer have therefore been performed (Adams and Wong, 1968; Abul-Hajj, 1975; Miller *et al.*, 1981). Our main findings are summarized

in Fig. 1. All tumours interconvert androgen precursors "on site". Inactive substrates such as dehydroepiandrosterone sulphate, present in breast fluids in large amounts (Miller *et al.*, 1980), may be metabolized into biologically-active products. About one-half of breast cancers have the potential to aromatize androgens into oestrogen. In most tumours the conversion is relatively small, but the activity is higher than that in adipose tissue incubated under the same

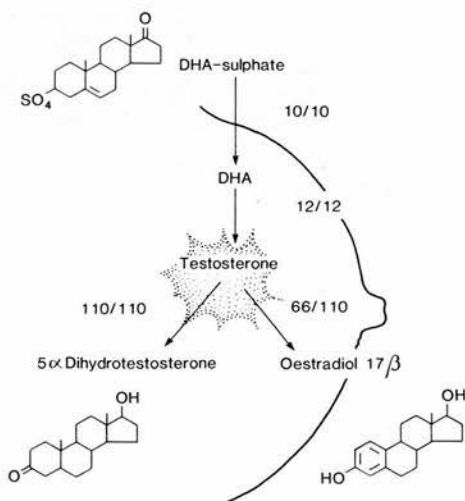


Figure 1. *In vitro* steroid metabolism by human breast cancers. The numbers represent tumours showing the particular metabolic conversion over the total tumours studied.

Aminoglutethimide — An alternative endocrine therapy for breast carcinoma, edited by R. W. Elsdon-Dew, I. M. Jackson and G. F. B. Birdwood, 1982: Royal Society of Medicine International Congress and Symposium Series No. 53, published jointly by Academic Press Inc. (London) Ltd., and the Royal Society of Medicine.

conditions (Abul-Hajj *et al.*, 1979; Perel *et al.*, 1980).

It is thus essential to elucidate the biological significance of tumour aromatization and determine whether it is related to the response to endocrine treatments, such as aminoglutethimide, which might be expected to be active against aromatase activity. For this purpose, three studies have been performed:

- (1) To confirm that aminoglutethimide will inhibit aromatase activity *in vitro*.
- (2) To determine the relationship between tumour potential for aromatization and oestrogen receptors, which are of established value in predicting hormone sensitivity (McGuire *et al.*, 1975).
- (3) To correlate these results with the clinical response of patients with advanced breast cancer treated with aminoglutethimide.

Investigations and Findings

To determine whether tumour aromatization was affected by aminoglutethimide, triplicate portions of 12 human breast cancers were incubated with ($7\alpha^3\text{H}$) testosterone for 2 h at 37°C in the presence or absence of 0.01 and 0.1 mmolar concentrations of aminoglutethimide. Seven tumours synthesized oestradiol, and aminoglutethimide inhibited production in all of them (Fig. 2). In no incubation was oestradiol synthesis completely abolished but inhibitions of between 60 and 85% of control values were observed at 0.1 mmol concentration.

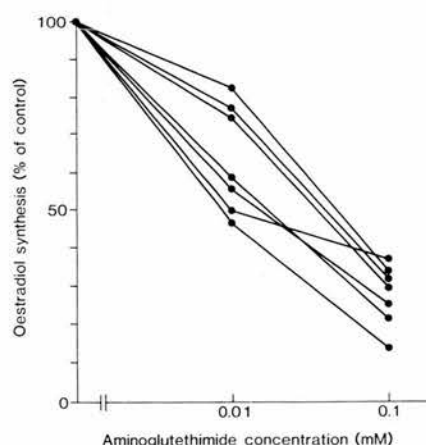


Figure 2. Inhibition of aromatase in human breast cancer by aminoglutethimide at two concentrations, as shown by reduction in oestradiol synthesis.

To investigate the relationship between tumour aromatization and hormone sensitivity we have measured cytoplasmic oestrogen receptors and conversion of ($7\alpha^3\text{H}$) testosterone to oestradiol in 110 human breast cancers. Evidence for oestradiol synthesis (conversion $> 0.02\%$ of the precursor) was obtained in 66 and for oestrogen receptor activity (> 5 fmol/mg cytosol protein) in 81 tumours. The relationship between presence of receptor and potential for aromatization is shown in Table 1. Whereas most receptor-positive tumours synthesized oestradiol, most receptor-negative tumours did not. This trend for aromatization to be associated with receptor-positive tumours was statistically significant. Additionally, levels of oestrogen receptor were significantly higher in tumours with oestrogen biosynthesis compared with those lacking aromatization.

Table 1

Relationship between potential for tumour oestrogen synthesis and presence of oestrogen receptor

Tumour	Oestrogen synthesis	Without synthesis
Receptor-positive	55	26
Receptor-negative	11	18

$\chi^2 = 7.99$ $P < 0.005$

Because of this finding, it is tempting to speculate that tumours which not only have receptors for oestradiol, but also possess the potential to synthesize oestradiol, might have a greater need for hormones than tumours without synthesis and/or receptors. If so, treatments such as aminoglutethimide which inhibit aromatization might be more effective in patients whose tumours possess aromatase activity.

We have therefore measured tumour oestrogen biosynthesis and receptors in patients with advanced breast cancer subsequently treated with aminoglutethimide. The results have been correlated with the clinical response of the patient (Table 2). The number of women studied is still small, but tumours with highest *in vitro* conversion to oestradiol and which also possessed oestrogen receptors were those which responded to aminoglutethimide while tumours without aromatizing potential or oestrogen receptors failed to respond. It is therefore possible that screening for aromatase activity in receptor-positive tumours might permit selection of patients most likely to respond to aminoglutethimide.

Table 2

Relationship between tumour oestrogen biosynthesis (percentage conversion of (7 α ³H) testosterone to oestradiol), oestrogen receptor and clinical response to aminoglutethimide

Patient	Oestrogen biosynthesis	Oestrogen receptor	Clinical response
A	0.18%	+	Yes
B	0.09%	+	Yes
C	0.08%	+	Static
D ^a	0.03%	+	Mixed
E ^a	Not detectable (<0.02%)	+	No
F		+	No
G ^a		-	No
H		-	No

^aIn combination with tamoxifen

Conclusion

It seems likely that the benefits of aminoglutethimide treatment in postmenopausal women with advanced breast cancer are achieved not only by blocking adrenal production of hormones at the level of cholesterol side-chain cleavage but also by a more selective action against peripheral aromatization of androgens to oestrogen by adipose and tumour tissue. Evidence from the interrelationship with oestrogen receptors and, in a limited number of patients, response to aminoglutethimide suggest that tumour biosynthesis may have clinical significance. It seems logical to treat tumours which have both oestrogen receptors and the potential to synthesize oestrogen for the receptor, with regimes which inhibit the aromatase process.

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Discussion

Asked by Dr R. Paridaens (Brussels) whether the data had been analysed as a function of menopausal status, Dr Miller said that all the patients

studied for aminoglutethimide effects were postmenopausal. In the work on oestrogen receptors and biosynthesis, a few premenopausal patients had been included but most were postmenopausal in this group too.

Steroid Metabolism and Oestrogen Receptors in Human Breast Carcinomas

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Abstract—Metabolism of 7α [^3H] testosterone and oestrogen receptor activity have been measured in 54 human breast cancers. All tumors converted testosterone to $\Delta 4$ androstenedione, 5α dihydrotestosterone and 5α androstenediol, but unequivocal evidence for production of oestradiol was obtained only 29 of the tumours. Thirty-seven tumours were classified as oestrogen receptor 'positive' containing levels in excess of 5 fmol/mg cytosol protein. Although mean conversions to $\Delta 4$ androstenedione, 5α dihydrotestosterone and 5α androstenediol were all higher in oestrogen receptor negative tumours as compared with receptor positive group, the differences did not reach statistical significance. There was, however, a significant trend for oestradiol synthesis to be associated with oestrogen receptor positive tumours ($P < 0.025$). All tumours with very high level of receptors converted testosterone to oestradiol.

INTRODUCTION

HUMAN breast cancer transforms C_{19} steroid precursors into active androgens and oestrogens. Thus dehydroepiandrosterone and its sulphate are metabolised *in vitro* to testosterone[1-3], oestrone[1,3] and oestradiol- 17β [4]. $\Delta 4$ androstenedione is converted to testosterone, oestrone and oestradiol- 17β [5]; and testosterone metabolised to 5α dihydrotestosterone, 5α androstenediol[2,6] and oestradiol- 17β [7-9].

Synthesis of oestradiol from androgen precursors of adrenal origin by breast cancers may be of importance in response of tumours to endocrine treatments, particularly in postmenopausal patients. In these women in whom circulating oestrogen levels are low, tumour metabolism may provide a supply of oestrogen required for tumour growth. Currently the oestrogen receptor activity of a tumour is the best index of its likely hormone sensitivity (for recent review[10]), and the present study of 54 tumours was undertaken to determine whether oestrogen receptor activity and tumour steroid metabolism were related.

MATERIALS AND METHODS

Patients

Fifty-four histologically proven cancers of the breast from 8 premenopausal, 2 early menopausal (less than 5 years since the last

menstrual period) and 44 postmenopausal women were studied. Thirty-nine tumours were primary carcinomas and 15 local recurrence.

Following excision, tumours were immediately placed on ice. Sufficient tissue was removed for histopathological diagnosis and the remainder used to determine steroid synthesis and oestrogen receptor activity.

Steroid metabolism

A portion of each tumour (0.5 g) was finely sliced and incubated for 2 hr at 37°C in Krebs Ringer phosphate buffer pH 7.4 containing an NADPH generating system and $22.5 \mu\text{Ci}$ [7α - ^3H] testosterone (8.9 Ci/mmol). The reaction was stopped by addition of methanol (40 ml) and the incubate stored at -10°C until further processing. Before extraction of the metabolites, 500 μg of non-radioactive carrier of the steroids to be characterised were added to monitor losses. The metabolites were then extracted, separated into individual steroids, purified by thin layer chromatography and characterised by the methods previously described[8, 11]. Characterisation of individual metabolites was by chemical derivative formation and based on (a) similar chromatographic mobilities of parent and derivatized steroids with authentic standards (b) consistent specific radioactivity throughout these procedures. Metabolism was determined by measuring the percentage of radioactivity in the

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various characterised metabolites after correction for procedural losses. Conversions in excess of 0.02% are detectable by these methods. Control incubations performed in the absence of tumour showed negligible metabolism apart from the production of small amounts of $\Delta 4$ androstenedione (<0.1%).

Oestrogen receptors

The concentration of oestrogen receptor was determined by saturation analysis [12]. Tumour cytosol was incubated overnight at 4°C with 10 pg [2,4,6,7³H] oestradiol-17 β and varying amounts of non-radioactive oestradiol-17 β (0, 10, 30, 70, 90 and 20,000 pg). Separation of free and bound steroids was by addition of dextran-coated charcoal, the protein bound fraction being determined by liquid scintillation counting. After correction for non-specific binding, the data was analysed according to Scatchard [13] to derive concentrations of receptor sites. Activities in excess of 5 fmol/mg cytosol protein were regarded as receptor positive in accordance with recommendations of the British Breast Group Study Group [14].

RESULTS

All tumours metabolised 7 α [³H] testosterone to $\Delta 4$ androstenedione, 5 α dihydrotestosterone and 5 α androstenediol. Evidence for aromatization to oestradiol-17 β was obtained in 29 tumours (54%); the remaining tumours showed

no evidence for oestradiol synthesis. Conversion to oestrone was investigated in several tumours but its production when detected was always less than oestradiol and only present in tumours which synthesized oestradiol.

Metabolism of testosterone to $\Delta 4$ androstenedione, and 5 α dihydrotestosterone (but not 5 α androstenediol) in tumours from premenopausal women was significantly higher than in those from postmenopausal patients (Table 1). The proportion of tumours showing oestrogen synthesis and level of that synthesis were similar in both menopausal groups.

Thirty-seven (68.5%) tumours were oestrogen receptor positive, the remainder being negative. The proportion of oestrogen receptor positive tumours was similar in pre and postmenopausal patients but the concentration of receptor in positive tumours was lower in the 5 premenopausal (Mean 33.4 fmol/mg cytosol protein) than in the 31 postmenopausal patients (Mean 144.6 fmol/mg cytosol protein).

Although the mean conversions of testosterone to $\Delta 4$ androstenedione, 5 α dihydrotestosterone and 5 α androstenediol were higher in oestrogen receptor negative tumours as compared with receptor positive group, the differences did not reach significance (Table 2).

However, there was a significant relationship between the capacity to synthesise oestradiol from testosterone and oestrogen receptor activity (Table 3). Thus the majority of oes-

Table 1. Metabolism of 7 α [³H] testosterone in tumours from pre- and postmenopausal patients

Metabolite	% Conversion (Mean \pm S.D.)		Significance*
	Premenopausal (8)	Postmenopausal (44)	
$\Delta 4$ androstenedione	4.83 \pm 3.46	4.10 \pm 8.84	P < 0.01
5 α dihydrotestosterone	3.62 \pm 2.11	1.46 \pm 1.24	P < 0.01
5 α androstenediol	1.94 \pm 2.51	0.82 \pm 0.81	NS
oestradiol-17 β	0.11 \pm 0.08(4)	0.09 \pm 0.07(23)	NS

*By Wilcoxon Rank Test.

NS = Not significant.

Table 2. Metabolism of 7 α [³H] testosterone in oestrogen receptor positive and negative breast carcinomas

Metabolite	% Conversion (Mean \pm S.D.)		Significance
	Oestrogen receptor + ve (37)	Oestrogen receptor - ve (17)	
$\Delta 4$ androstenedione	2.43 \pm 3.05	7.78 \pm 12.85	NS
5 α dihydrotestosterone	1.54 \pm 1.40	2.96 \pm 3.29	NS
5 α androstenediol	0.74 \pm 0.75	1.67 \pm 1.86	NS

NS = Not significant by Wilcoxon Rank Test.

Table 3. Correlation between potential for oestradiol synthesis and presence of oestrogen receptor

	Oestrogen synthesis	Without synthesis
OE ₂ R + ve	24	13
OE ₂ R - ve	5	12

$$\chi^2 = 5.89 \quad P < 0.025$$

trogen receptor-positive tumours synthesised oestradiol from testosterone, whereas most oestrogen receptor-negative tumours did not. All tumours with receptors in excess of 120 fmol/mg cytosol protein synthesised oestradiol (Fig. 1).

Mean level of oestrogen receptor in tumours which synthesised oestradiol was also significantly higher than that in those without synthesis.

In those tumours with aromatizing activity the actual amounts of oestradiol synthesised did not relate to receptor status and in those tumours which possessed both activities, levels of oestradiol synthesis were not related to the concentration of oestrogen receptors in the tumour (Fig. 2).

DISCUSSION

The results confirms our other previous findings[8, 15] that all human breast cancers metabolise testosterone to 5 α dihydrotestos-

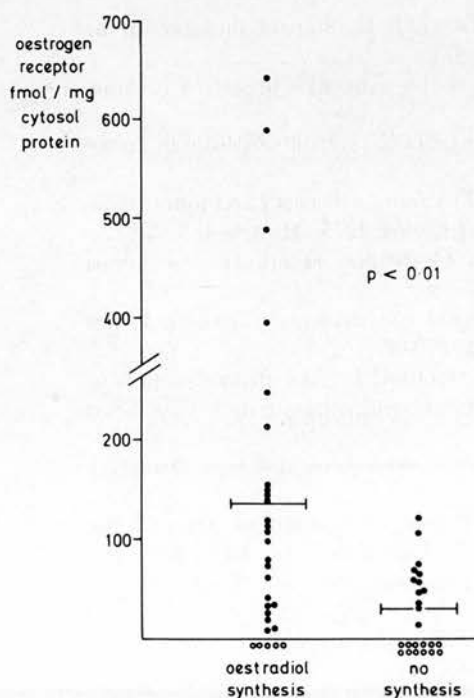


Fig. 1. Level of oestrogen receptors in tumours with and without oestradiol synthesis. The difference between groups is significant by Wilcoxon Rank Test ($P < 0.01$).

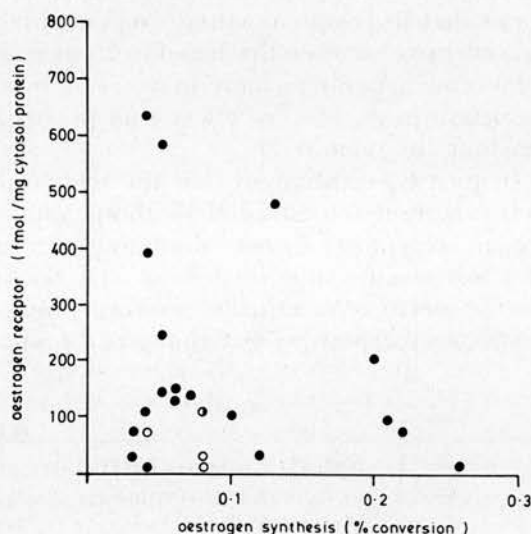


Fig. 2. Relationship between level of oestrogen receptors and % conversion to oestradiol in tumours with both activities ○ premenopausal; ◐ menopausal; ● postmenopausal.

terone, 5 α androstenediol and $\Delta 4$ androstenedione but only a portion of tumours can synthesise oestradiol-17 β . The incidence of oestrogen receptor positive tumours (68.5%) is also consistent with previous reports (review[10]).

No significant relationship was detected between tumour conversion of testosterone to C₁₉ steroid metabolites and oestrogen receptor activity, but there was a significant correlation between receptors and the capacity to synthesise oestradiol from testosterone. Tumours with oestrogen receptor activity, particularly when the level was high, were more likely to possess aromatizing enzymes. This finding differs from two recent reports; but the number of tumours reported in them was small[5, 16]. In a single report an inverse correlation between oestrogen receptors and synthesis has been described[4], but only 18 tumours were studied and the incidence of receptor positive tumours and the concentration of receptor sites were lower than that generally found[10]. In particular there was an absence of tumours with high receptor concentration. This may be relevant as in the present study these were most likely to synthesise oestradiol.

The finding of a positive correlation between oestradiol synthesis by a tumour and its receptor status suggests that the ability of a tumour to synthesise oestrogen may be related to its hormone responsiveness.

The recent observation that the MCF7 cell line of human breast cancer could synthesise sufficient oestrogen from extracellular C₁₉ steroid to maintain oestrogen-dependent events[17] would support our previous hypothesis that in postmenopausal women with advanced breast cancer the benefit of adrenalectomy and hypophysectomy may result from a reduction in the level of C₁₉ steroid precursors reaching the tumour[7].

It must be emphasized that the relationship between tumour oestradiol synthesis and oestrogen receptors is not absolute; oestrogen receptor positive tumours may or may not synthesise oestrogen. Equally, however, not all oestrogen receptor positive tumours respond to

endocrine ablation[18,19]. It is possible that if a tumour not only has receptor sites for oestrogen but also possesses the capacity to synthesize oestradiol, it may be particularly sensitive to oestrogen or at least have a greater need for oestrogen than tumours without biosynthetic potential. (Paradoxically, however, a tumour with both receptors and aromatase activities might appear less dependent upon circulating oestrogens, being in fact more dependent upon circulating androgens). If this is so, then screening for aromatase activity in receptor-positive tumours might permit selection of patients who are likely to respond to endocrine therapy, particularly to regimes such as adrenalectomy, which removes the source of circulating androgens, or administration of aminoglutethimide and other compounds, which inhibit aromatisation[20,21]. Clinical studies are being conducted to determine these relationships.

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Tumour steroid hormone synthesis and estrogen receptor status in breast cancer patients

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Keywords: breast cancer, estrogen receptors, steroid biosynthesis

Summary

The capacity of breast cancer to synthesise active androgens and estrogens has been related to estrogen receptor (ER) status in 79 postmenopausal patients with breast cancer. Although there was no quantitative relationship between levels of ER and steroid metabolism in ER positive tumours, there was (a) a positive correlation between estrogen synthesis and ER positivity and (b) increased androgen synthesis and ER negativity. This may imply an inherent difference in the handling of hormones in ER positive and negative tumours.

Introduction

The role of ER status in predicting the hormonal sensitivity of breast cancer is well established (1). So also is the ability of breast cancer tissue to metabolise and aromatise C19 steroids to form estrogens (2, 3). In our study of 54 breast cancers, all tumours have shown the enzymatic activity to convert testosterone to its 5 α reduced products but only in 29 has aromatisation been demonstrated by the synthesis of estradiol 17 β (4).

The interrelationship of these two activities has also been investigated by ourselves and others but with conflicting results (4-9). In this study we have attempted to clarify the relationship by extending our original number of tumours.

Methods

The breast cancers studied were obtained from 79 postmenopausal women with primary cancer of the breast. The removed tumour and/or the breast containing it was placed on ice in the operating theatre and immediately transferred to the laboratory. Following removal of sufficient tumour for histological examination, a portion was dissected free of extraneous fat and other tissue and divided for assays

of ER activity and steroid metabolism.

ER activity was determined by saturation analysis (10). A portion of tumour (300 mg) was homogenised in 1 mM Tris monothioglycerol buffer (1:5 w/v) and centrifuged at 3200 rpm for 20 mins. Aliquots of the resultant cytosol were incubated overnight at 4°C with 10 pg of 2467³H estradiol and varying amounts of competing non-radioactive estradiol (0, 10, 30, 70, 90 and 20,000 pg). Bound steroid was separated from free by the addition of dextran-coated charcoal and the bound fraction measured after centrifugation by liquid scintillation counting. The concentration of receptors was determined from a 6-point Scatchard analysis (11). Cytosol protein was determined by the method of Bradford (12) and receptor levels in excess of 5 fmol/mg tumour cytosol protein were regarded as indicating an estrogen receptor positive tumour.

Steroid metabolism: tumour (500 mg) was finely sliced and incubated for 2 hours at 37°C in Krebs Ringer phosphate buffer pH 7.4 containing an NA-DPH generating system and 22.5 μ Ci 7 α -³H testosterone (8.9 Ci/mmol). The reaction was terminated by the addition of methanol and the incubate stored at -10°C until further processing. Before extraction of the metabolites, 500 μ g of non-radioactive carrier steroid were added for each metabolite to be purified so that procedural losses could

serially transplanted chemically induced rat mammary tumours. With passage there is loss of hormone dependence and estrogen receptor activity along with an increase in 5α reduction (14).

The picture which emerges is that tumours possessing estrogen receptor protein have an increased tendency towards synthesising estrogen from C19 steroids. Conversely, those tumours without estrogen receptor activity tend to synthesise larger amounts of active androgens. Whether the differences in steroid metabolism as observed in vitro also occur in vivo remains to be shown. It is also possible that different levels of endogenous precursor may be present in tumours and this may influence steroid metabolism. Equally the clinical relevance of this relationship between steroid receptors and metabolism still remains to be defined but it could be that inhibitors of peripheral aromatisation, e.g. aminoglutethimide (15), could be particularly valuable in those tumours showing both receptor activity and estrogen synthesis.

Acknowledgment

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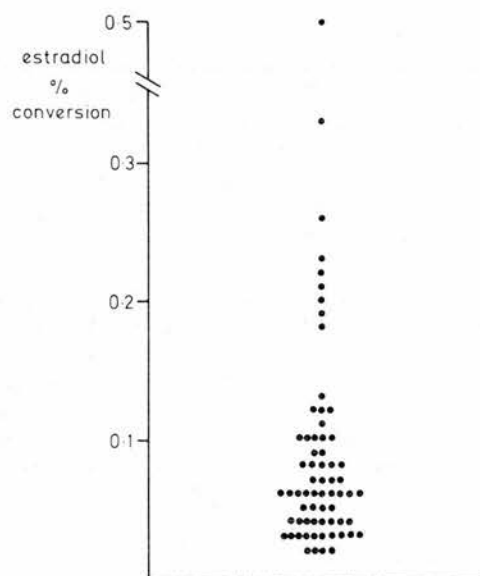


Chart 1. Level of estradiol synthesis in tumors with aromatization.

Table 1
Correlation between potential for aromatization and presence of estrogen receptor

 $\chi^2 = 7.99; p = 0.005.$

	Estrogen synthesis	Without synthesis
Estrogen receptor positive	55	26
Estrogen receptor negative	11	18

solute. This effect was largely accounted for by the association of aromatase with estrogen receptors, all tumors with aromatase activity responding to treatment also being estrogen receptor positive.

It was also of interest to examine the relationship between tumor aromatization and response to specific therapies which might be active against aromatization. The results are presented in Table 2, both for adrenalectomy, which removes the major source of C-19 steroid precursors in postmenopausal women, and for the administration of aminoglutethimide, which also inhibits tumor aromatase (2).

Although numbers are small, tumors with the greatest *in vitro* conversion to estradiol were those which responded while those without aromatization failed to do so.

Discussion

Our report that human breast cancer may synthesize estrogen from C-19 steroid precursors (13) has been confirmed by several groups (1, 4, 7, 24). When compared with ovarian production in premenopausal women, the levels of estrogen synthesized by a tumor may appear insignificant. However, in postmenopausal women with breast cancer, a tumor may represent a major estrogen-synthesizing organ. Using identical conditions of *in vitro* incubation, we have found that breast cancers may have higher aromatase activity than do adrenal cortex, liver, or fat.²

Fat is generally assumed to be the major site of peripheral conversion of androgens to estrogens (10, 21). However, direct

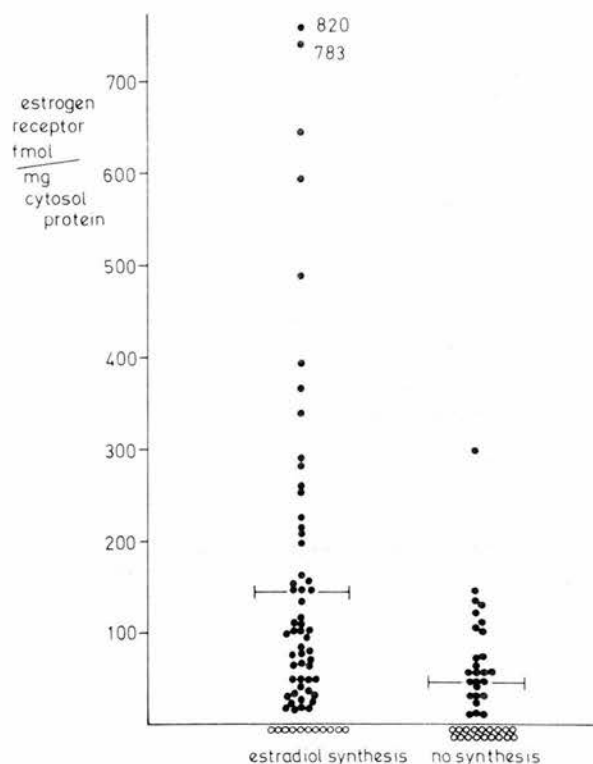


Chart 2. Level of estrogen receptor in tumors with and without estradiol synthesis. The difference between groups is significant by Wilcoxon rank test ($p < 0.001$).

comparisons of adipose tissue and breast cancer have always shown higher biosynthesis of estrogen in tumor (3, 22). Furthermore, using the methods of the present studies, biosynthesis of estrogens from dehydroepiandrosterone, Δ^4 -androstenedione, and testosterone was consistently below detectable levels in adipose tissue. The large mass of fat in the body may compensate for its low synthetic activity and make it the major source of circulating estrogen in postmenopausal women. However, "on site" tumor production of estrogen may be more important for the growth of the breast cancer.

It has been calculated that the MCF-7 cell line of human breast cancer might synthesize sufficient intracellular estrogen to stimulate estrogen-mediated events (11). In the present studies, similar calculations indicate that 0.5 to 12.5 pmol are formed during incubation. This is sufficient to half-saturate estrogen receptor sites in the majority of breast cancers. However, it should be noted that endogenous levels of testosterone in breast tumors are likely to be considerably lower than are those used in our incubations (20). On the other hand, in the breast there are high concentrations of other C-19 steroid precursors (18) which breast tumors may convert to testosterone or metabolize directly to estrogen (1, 7, 17, 19).

In addition to these theoretical considerations, it is important to relate the results from *in vitro* incubations to estrogen receptor status which has already been established to be of clinical value (12).

The finding of a positive correlation between aromatase and estrogen receptor activity confirms our previous report (17) but is not in agreement with others (3, 9, 24). However, in these latter studies, smaller numbers were reported. This may be important, since the relationship between aromatase and re-

² W. R. Miller, Unpublished observations.

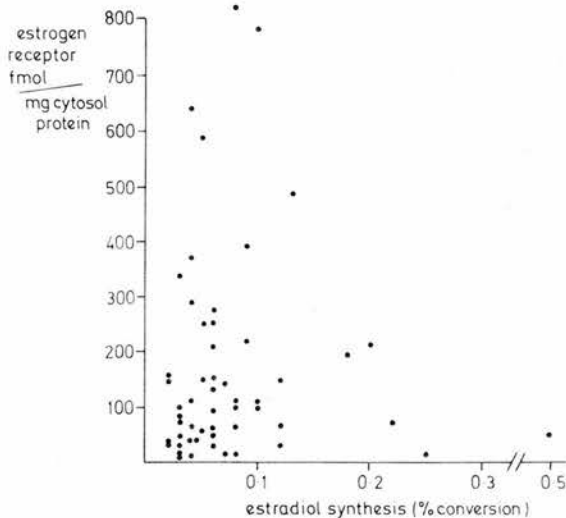


Chart 3. Relationship between level of estrogen receptors and percentage of conversion to estradiol in tumors with both aromatase and receptor activities.

40 patients with advanced breast cancer
13 responders to endocrine therapy (32.5%)

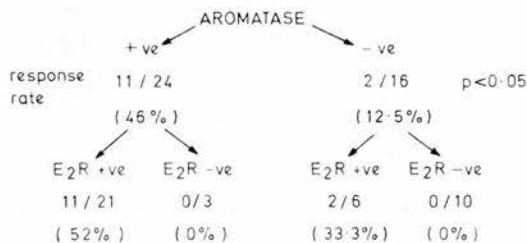


Chart 4. Relationship between tumor aromatization, estrogen receptors, and response to endocrine therapy.

Table 2

Relationship between tumor aromatization and response to either aminoglutethimide or adrenalectomy

	Patient	Aromatase ^a (%)	Estrogen receptor	Response
Aminoglutethimide	E. M.	0.18	+	Yes
	K. S.	0.09	+	Yes
	J. S.	0.08	+	Static
	B. E. ^b	0.03	+	Mixed
	I. T. ^b	ND ^c	+	No
	I.H.	ND	—	No
Adrenalectomy	J.B.	ND	—	No
	J.R.	ND	—	No

^a Aromatase activity expressed as percentage of conversion of [7 α -³H]testosterone to 17 β -estradiol.

^b In combination with tamoxifen.

^c ND, not detectable, i.e., <0.02%.

ceptors is not absolute and the trend may not be statistically significant when small numbers of tumors are investigated (16). Our finding is logical in that tumors which synthesize estrogen would be expected to possess the mechanism (6) by which it gains access to the nucleus and exerts its effect.

We postulated that tumors possessing both estrogen-synthetic and receptor activity were more likely to be sensitive to deprivation of hormones than were those without one or both of these properties (17). The clinical studies that we now report do not totally support this view. Although there did appear to

be a positive relationship between aromatase activity and clinical response, this was neither additive to, nor independent of, estrogen receptor activity. The relationship between tumor aromatase activity and endocrine responsiveness may be best shown by studying those forms of treatment which deprive the tumor of its precursor C-19 steroids or directly inhibit tumor aromatase. These include adrenalectomy and aminoglutethimide, and it is encouraging to note that tumors with highest aromatase activity appeared to be more responsive to these therapeutic methods. However, the numbers of patients studied are small, and it is now important to establish a prospective study to determine the relationships between aromatase activity, steroid receptor, and tumor sensitivity to endocrine treatment.

It is difficult to attribute a physiological role for estrogen synthesis in tumors which lack estrogen receptor activity, although it has been suggested (1) that these tumors may appear hormone independent by virtue of their *de novo* synthesis. Further studies are required to elucidate this.

One can conclude that estrogen-synthetic activity can be demonstrated in approximately half of all breast cancers and in many is likely to be of sufficient magnitude to induce estrogen-stimulated events. Although aromatase is correlated with estrogen receptor activity, the relationship is not absolute. However, we have some evidence that estrogen synthesis may be an important influence on the hormonal sensitivity of a tumor, especially to regimens such as adrenalectomy and aminoglutethimide.

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biosynthesis. Measurements of hormones in adrenal arterial and venous blood indicate that the adrenal also synthesizes only small amounts of oestrogen. However, the adrenal does produce large amounts of androgen which can be potentially utilized as precursor for transformation into oestrogen elsewhere. The observation that in adrenalectomized, oophorectomized women, perfusion of androgens causes plasma oestrogen levels to rise, supports the contention that peripheral transformation of adrenal androgens represents the major route of oestrogen formation in postmenopausal women.

SUMMARY

A proportion of breast cancers require oestrogen for their continued growth. The primary sources of this oestrogen in post-menopausal women are peripheral sites whose aromatase activity transforms inactive adrenal androgens into active oestrogen. The development of inhibitors to block this peripheral activity represents a novel and potentially important form of therapy for breast cancer. In this respect, aminoglutethimide has already proven to be of value in the treatment of breast cancer and 4 hydroxy-androstenedione shows promise for the future. The presence of aromatase in certain patients makes breast cancer a useful model of peripheral aromatase and screening for the potential amongst breast cancers may help select patients for treatment with anti-aromatase agents.

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LEGENDS TO FIGURES

FIGURE 1

Pathway of oestrogen biosynthesis from cholesterol

FIGURE 2

The aromatase reaction

FIGURE 3

Aromatase inhibitors

FIGURE 4

Inhibition of aromatase by aminoglutethimide in human breast cancers

TABLE

Relationship between tumour aromatase and response to
aminoglutethimide

		Response	No Response
tumour	-ve	0	4
aromatase	+ve	8	2

$$\chi^2 = 6.56, p < 0.025$$

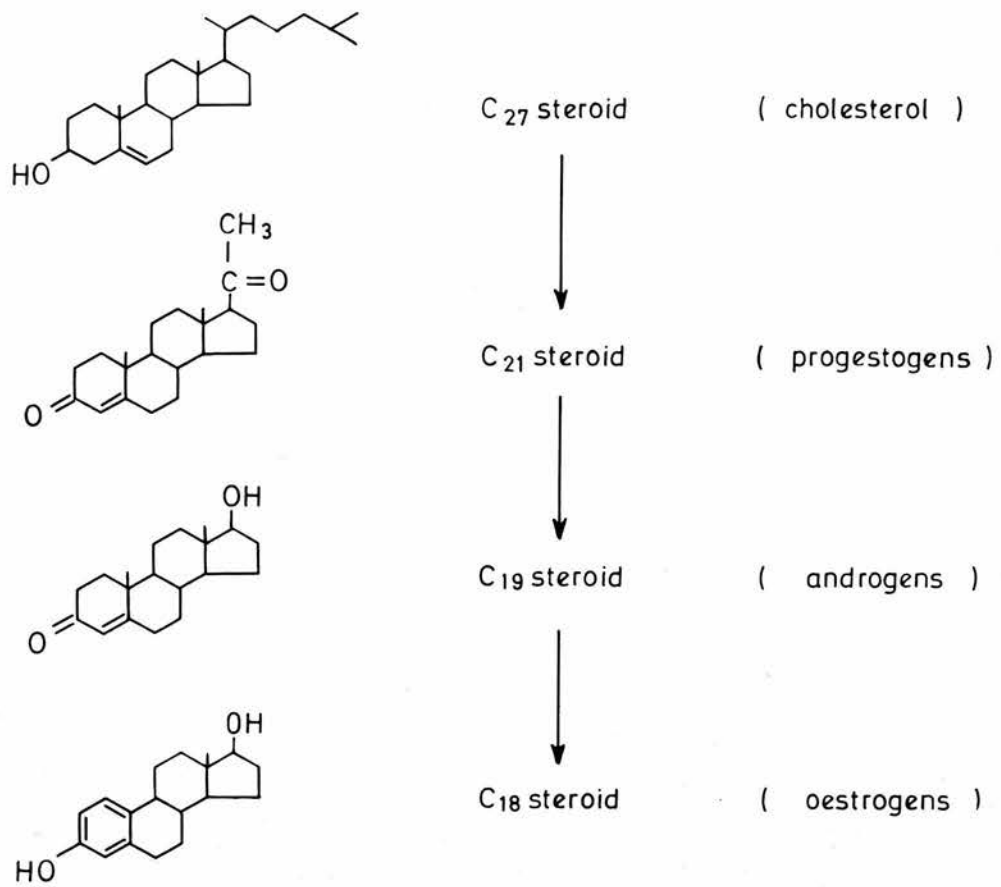


Figure 1

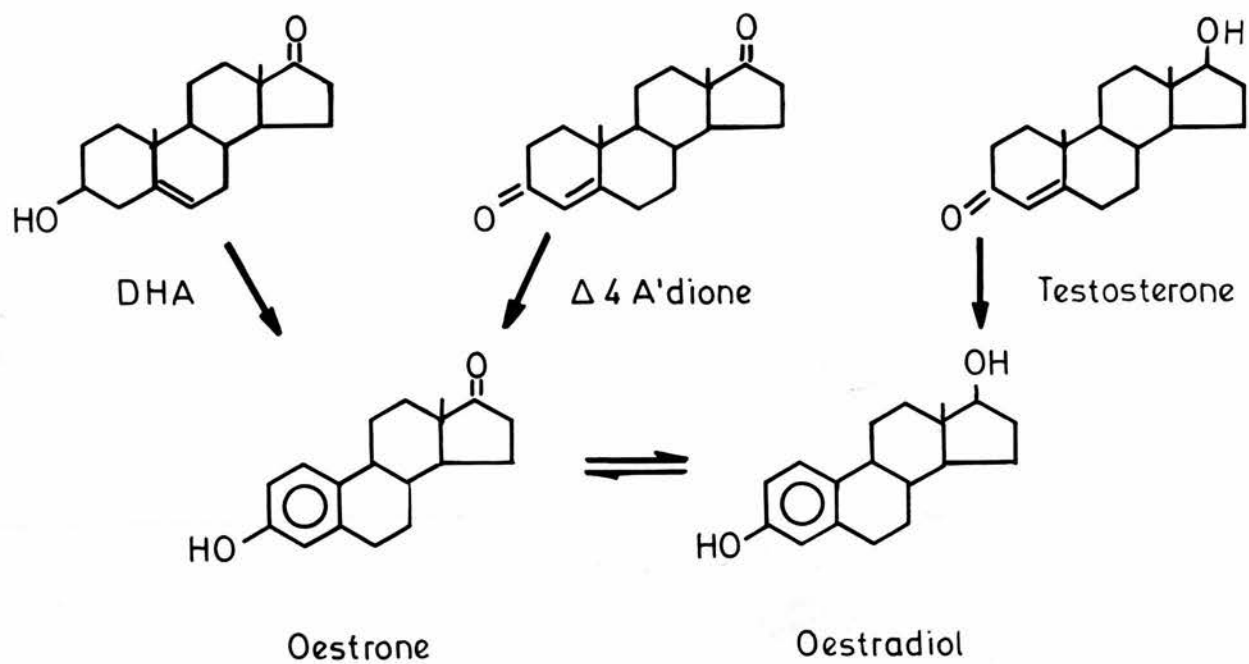
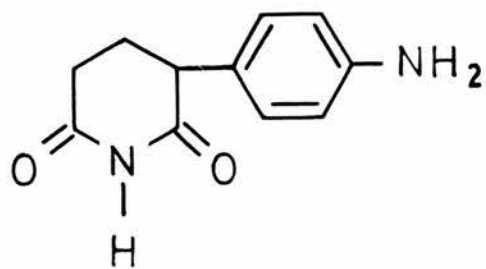
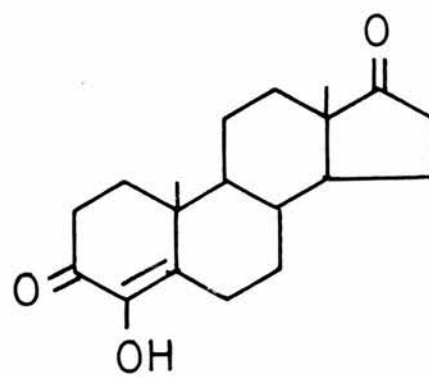


Figure 2



aminoglutethimide



4-hydroxy - androstenedione

Figure 3

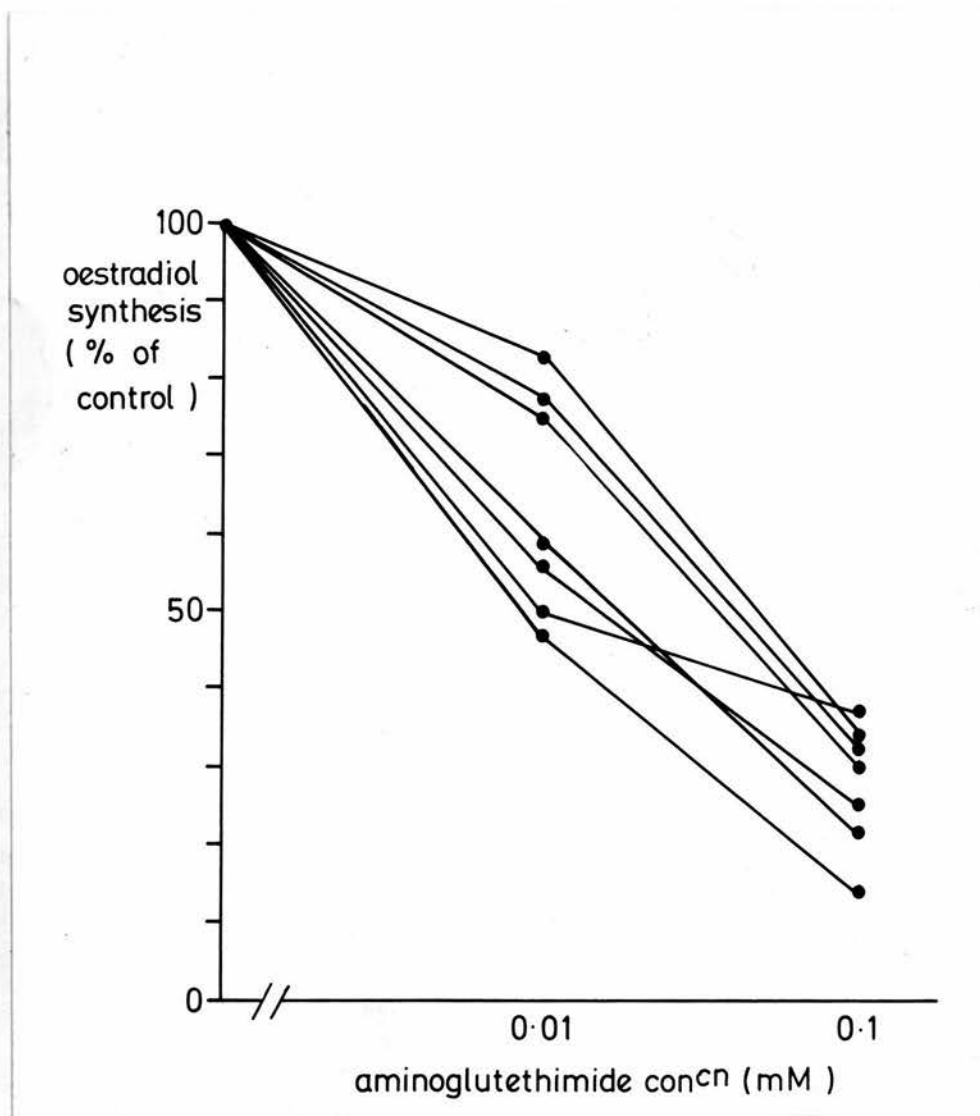


Figure 4

In Vitro Effects of Oestrogen on Testosterone Metabolism by Human Breast Cancers

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Abstract—The effect of *in vitro* addition of oestradiol on the metabolism of $7\alpha^3H$ testosterone by 15 human breast cancers has been investigated. Oestradiol significantly inhibited metabolism and conversion to 5α dihydrotestosterone, 5α androstenediol and Δ^4 androstenedione.

INTRODUCTION

HUMAN breast cancers can metabolise steroid precursors to active hormones such as 5α dihydrotestosterone [1, 2] and oestradiol- 17β [3, 4]. Whilst the metabolism of testosterone by rat mammary tumours has been shown to be influenced by oestrogen both *in vitro* [5] and *in vivo* [6], little is known about the effects of hormones on steroidogenesis in human tumours. The aim of the present study was to determine *in vitro* the effect of oestradiol- 17β on testosterone metabolism by human breast cancer.

MATERIALS AND METHODS

Tumours from 15 patients with proven cancer of the breast were studied: 10 subjects were at least 5 yr postmenopausal, 4 were premenopausal and had regular menstrual periods at the time of investigation. One patient had undergone bilateral oophorectomy 15 months previously.

Following excision the tumours, 10 of which were primary carcinomas and 5 recurrence on the chest wall, were processed at 0°C . They were finely sliced and split into duplicate portions each weighing 0.5 g to which Krebs Ringer phosphate buffer pH 7.4 (5 ml), an NADPH generating system and $20\ \mu\text{Ci}$ $7\alpha^3H$ testosterone (5.7 Ci/mmol from Radiochemical Centre, Amersham) were added.

Incubation was then carried out either in the presence of oestradiol ($1.5\ \mu\text{g/ml}$) or its vehicle (propyleneglycol:ethanol 1:1) by

shaking at 37°C in an atmosphere of oxygen for two hours. In two tumours oestradiol-3-methyl ether ($1.5\ \mu\text{g/ml}$) was added to a further incubate as an additional control. The reaction was stopped by the addition of methanol (40 ml) and the incubate stored at -10°C .

Before extracting the metabolites, $500\ \mu\text{g}$ each of non-radioactive testosterone, 5α dihydrotestosterone (5α DHT), 5α androstenediol, Δ^4 androstenedione and oestradiol- 17β were added to each incubate to monitor recovery losses. Details of the methodology for steroid purification by thin layer chromatography and characterisation by chemical derivative formation have been described previously [7].

Metabolism of testosterone was determined by measuring the incorporation of radioactive label into the appropriate metabolites after correction for recovery losses. Statistical comparisons between the results of incubates with and without added oestradiol were performed by paired *t*-tests after logarithmic transformation of the data.

RESULTS

The results are presented in Table 1. All 15 cancers converted testosterone to 5α DHT, 5α androstenediol and Δ^4 androstenedione but synthesis of oestradiol was detected in only 8 tumours. In certain tumours, these products represented only a small proportion of the total metabolites; uncharacterized highly-polar compounds being responsible for most of the unidentified material.

The addition of oestradiol- 17β to the incubates caused significant inhibition of the metabolism of testosterone ($P < 0.01$) and its

Table 1. In vitro effects of oestradiol 17β metabolism of $7\alpha^3\text{H}$ testosterone by human breast carcinomas

	Testosterone metabolised	5α DHT	Production of 5α Androstenediol	$\Delta 4$ Androstenedione	Oestradiol
	(ng)	(ng)	(ng)	(ng)	(ng)
1. Control	203.4	16.3	16.1	103.8	—
+OE2	102.3	12.9	9.1	7.8	—
2. Control	183.5	38.8	13.3	35.2	0.8
+OE2	107.5	17.4	7.5	23.1	0.6
3. Control	204.6	4.5	1.1	3.9	0
+OE2	120.0	3.7	1.0	2.0	0
4. Control	232.4	17.1	6.7	11.9	0.4
+OE2	209.2	8.9	2.1	7.8	0.4
5. Control	234.3	34.0	7.4	36.7	0.4
+OE2	258.8	20.6	2.4	3.7	0.2
6. Control	170.3	16.8	8.6	46.8	3.5
+OE2	188.5	12.0	7.5	33.9	2.0
7. Control	218.0	13.0	9.8	5.8	0
+OE2	213.4	6.4	3.1	4.2	0
8. Control	537.9	6.6	1.3	382.2	0.7
+OE2	365.7	7.0	1.3	188.3	0.8
9. Control	107.0	16.0	14.2	16.2	2.0
+OE2	69.4	13.9	15.3	8.4	1.7
10. Control	196.1	39.5	16.1	4.9	0
+OE2	214.8	29.6	9.6	2.4	0
11. Control	152.0	11.4	5.7	3.8	0
+OE2	129.0	9.7	2.7	4.0	0
12. Control	432.8	25.0	14.4	121.7	0.3
+OE2	381.0	17.8	19.1	95.3	0.3
13. Control	220.4	31.7	24.2	46.6	0
+OE2	152.4	29.8	14.3	18.0	0
14. Control	182.9	17.1	4.6	5.4	0.6
+OE2	214.9	8.8	2.1	3.9	0.5
15. Control	168.4	17.7	3.2	4.8	0
+OE2	68.0	30.6	7.1	2.6	0
Geometric control	211.8	17.4	7.3	19.8	
Mean +OE2	164.3	12.9	4.9	8.0	

conversion to 5α DHT ($P < 0.01$), 5α androstenediol ($P < 0.05$) and $\Delta 4$ androstenedione ($P < 0.01$) as compared with control incubates. No effect was observed on the synthesis of oestradiol but the small conversions observed make comparisons difficult.

Oestradiol-3-methyl ether had no effect on the metabolism of testosterone in the two tumours (patients 1 and 8) in which its effect was investigated.

DISCUSSION

These results indicate that oestradiol- 17β in general, inhibits the metabolism of testosterone by human breast cancer. However, there is considerable variation not only in its effect between tumours but also on the conversion of testosterone to individual metabolites in the same tumour. It is possible that differences in endogenous steroids within tumours may contribute to this variation. The concentration of oestradiol- 17β added to the incubates was, in physiological terms, high.

However, this was similar to that which has been used by others to study oestrogen sensitivity in human breast cancer [8] and pharmacological doses are used when treating patients with advanced breast cancer by oestrogen therapy.

That the effect of oestradiol- 17β is not a non-specific effect of any steroid is shown by the fact that the same concentration of oestradiol-3-methyl ether was without effect in the two tumours to which it was added. Similar effects of oestrogen have, however, been described on 5α reduction of C19 steroids in the human adrenal cortex and prostate [9–10].

Although one of us reported that oestradiol- 17β inhibited 5α reduction of testosterone in hormone dependent rat mammary tumours but not in two hormone independent tumours [5], there is no evidence for such a relationship in human tumours. If oestradiol's effect on steroid metabolism in human tumours were limited to those which are hormonally sensitive, one would not expect to observe

the general inhibitory effects described in this study. Further, in two patients with recurrent disease, endocrine treatment failed to induce remission despite demonstrable *in vitro* inhibition of 5α reduction by oestradiol- 17β (patients 2 and 14).

Nevertheless, this study does indicate that metabolism of testosterone by human breast cancers is not an autonomous process but as

in normal endocrine tissues, can be influenced by the addition of other hormones.

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Report

Effects of drugs associated with hyperprolactinemia on plasma steroids and on steroid receptors and metabolism in human breast cancerR.C. Mason^a, W.R. Miller^a, R.A. Hawkins^a, M.S. Brown^b, and A.P.M. Forrest^a^a University Department of Clinical Surgery; ^b Immunoassay Section, Department of Clinical Chemistry, Royal Infirmary, Edinburgh EH3 9YW, UK

Keywords: breast cancer, estrogen receptor, hyperprolactinemic drugs, prolactin, steroid biosynthesis, steroid hormones

Summary

Certain commonly taken pharmaceutical preparations induce increased levels of plasma prolactin. The effects of these drugs on (a) tumor steroid receptors and metabolism, and (b) plasma hormones and hormone binding proteins have been studied in postmenopausal women with breast cancer. Two groups have been compared, 18 patients on drug treatment for at least 2 months and 15 subjects with no history of drug ingestion. Patients taking medication had significantly higher levels of plasma prolactin compared with control women. No significant difference was observed between the groups with regard to the plasma concentrations of dehydroepiandrosterone (DHA) and its sulphate (DHS), testosterone, estrone, estradiol-17 β , sex hormone binding globulin (SHBG), and albumin. Similarly, no difference was observed between these two groups with regard to estrogen receptor (ER), progesterone receptor (PR), or androgen receptor (AR) levels in the tumors nor their ability to metabolize (7-³H) testosterone. It is considered that the ingestion of these drugs does not affect tumor mechanisms involving steroids.

Introduction

The number of prescriptions written annually in the United Kingdom for tranquilizers and hypnotics exceeds 50 000 000 (1). Included in their total are many for patients with breast cancer (2).

Several of these drugs are known to raise plasma prolactin (Table 1). Elevation of prolactin is known to promote the growth of rat dimethylbenzanthracene (DMBA) induced tumors, to influence metabolism of steroids by these neoplasms (13), and to alter the concentration of estrogen receptors in cell cultures of human breast cancer (14). Prolactin may also have a role in the control of secretion of adrenal androgens, especially DHA and DHS (15),

which have been reported to be associated with the risk of breast cancer in humans (16). We have therefore investigated patients with breast cancer and studied the effects of those drugs which are known to stimulate prolactin secretion on the levels of circulating steroid hormones and on steroid receptors and steroid metabolic activity in the tumor.

Materials and methods*Patients*

Thirty-three postmenopausal women (at least 3 years from their last menstrual period) with pri-

Table 1. Drugs associated with stimulation of plasma prolactin.

Drug	Reference
<i>Anti-hypertensive agents</i>	
A methyl dopa	3
Rauwolfia alkaloids	3
<i>Tranquilizers</i>	
Phenothiazines	4
Haloperidol	5
Tricyclic antidepressants	3
Benzodiazepines	6
Meprobamate	7
<i>Others</i>	
Metoclopramide	8
Cimetidine	9
Estrogens	10
Contraceptive pill	11

mary breast cancer were studied. None had received any previous treatment for their disease. There was no evidence of neoplasia elsewhere or of endocrine disease. The women were subdivided into two groups according to their history of taking drugs. In group 1 were 18 women who had been taking the drugs listed in Table 2 for at least 2 months; in group 2 were 15 women who had no history of drug ingestion in the previous 6 months. Age and disease stage were not significantly different between these two groups of women (Table 3).

Plasma hormones and hormone binding proteins

Peripheral venous blood samples were obtained from all patients between 5 and 7 days before mastectomy at 30 min intervals between 9.30 am. and 11 am. via an indwelling cannula. The samples were immediately centrifuged, and the plasma separated and stored at -20°C until analysis. Assays were carried out between 3 and 12 months after collection. Prolactin was measured using a double antibody radioimmunoassay (17) modified for use with the Kemtek 3000 automated radioimmunoassay system (18). To minimize any effects of stress, the fourth (11.00 hr) plasma samples were assayed and results were expressed in mU/l of IRP75/504.

DHS was assayed using a direct radioimmunoassay (19) employing an antiserum raised against DHA, which cross-reacts with both DHA and DHS; since levels of DHS are at least 300 times

greater than DHA, the assay effectively measures plasma DHS. Samples collected at 10.00 hr and 10.30 hr were assayed and the mean results were expressed in ng/ml.

DHA was measured using the same radioimmunoassay following ether extraction from the plasma and back extraction with 0.1 M sodium hydroxide. The assay was validated by recovery of DHA and DHS added to standard and unknown plasmas. Recovery of DHA (80–85%) provided a check on procedural losses and that of DHS ($<0.01\%$) a check on the efficiency of differential extraction.

Testosterone was estimated using direct radioimmunoassay as described by Hillier et al. (20) but with omission of the chromatography step. Samples obtained at 10.00 hr and 11.00 hr were assayed and the mean results were expressed in nmol/l.

Estrone was assayed by ether extraction of the plasma, backwashing with 8% (w/v) sodium bicarbonate solution, and radioimmunoassay employing an antibody raised against estrone 6-carboxymethyloxime conjugated to albumin. The sample obtained at 10.30 hr was used for this assay and the results were expressed in ng/100 ml.

Estradiol was assayed by ether extraction of plasma made 30 mmol/l with regard to sodium hydroxide, and radioimmunoassay using an antibody raised against estradiol-17 β carboxymethyl-oxime conjugated to albumin. Samples obtained at 10.30 hr were also used for this assay and results were expressed in ng/100ml.

SHBG was measured by saturation analysis with precipitation of the bound protein by ammonium

Table 2. Drugs taken by patients in the study.

Drug	No. of patients
A methyl dopa	7
Diazepam	2
Meprobamate	2
Amitriptyline	1
Dothiepin hydrochloride	1
Chlorpromazine and amitriptyline	1
Cisflupenthixol decanoate	1
Haloperidol	1
Phenobarbitone	1
Phenobarbitone and diazepam	1

Table 3. Clinical and histological data.

Patient	Age	TNM	Pathology of tumor	Histology of nodes
<i>Drug patients</i>				
1	60	T ₃ N ₀	Invasive ductal carcinoma	negative
2	79	T ₂ N ₀	Invasive ductal carcinoma	negative
3	67	T ₂ N ₀	Invasive ductal carcinoma	negative
4	71	T ₃ N ₀	Undifferentiated carcinoma	positive
5	80	T ₂ N _{1b}	Invasive ductal carcinoma	negative
6	75	T ₄ N _{1b}	Adenocarcinoma	positive
7	62	T ₄ N _{1b}	Undifferentiated carcinoma	positive
8	72	T ₂ N _{1b}	Invasive ductal carcinoma	positive
9	69	T ₂ N _{1b}	Anaplastic carcinoma	positive
10	64	T ₄ N ₂	Scirrhus carcinoma	positive
11	74	T ₃ N ₃	Adenocarcinoma	positive
12	65	T ₂ N _{1b}	Invasive ductal carcinoma	positive
13	59	T ₂ N ₀	Scirrhus carcinoma	negative
14	56	T ₂ N ₀	Invasive ductal carcinoma	negative
15	57	T ₂ N ₀	Adenocarcinoma	negative
16	51	T ₂ N _{1b}	Invasive ductal carcinoma	positive
17	75	T ₂ N ₀	Lobular carcinoma	positive
18	66	T ₂ N _{1b}	Small cell carcinoma	positive
<i>Control patients</i>				
19	72	T ₁ N ₀	Undifferentiated carcinoma	negative
20	66	T ₂ N ₀	Invasive ductal carcinoma	positive
21	65	T ₂ N ₀	Undifferentiated carcinoma	negative
22	57	T ₂ N ₀	Undifferentiated carcinoma	negative
23	59	T ₂ N ₀	Invasive ductal carcinoma	negative
24	75	T ₄ N ₃	Anaplastic carcinoma	positive
25	67	T ₂ N ₀	Anaplastic carcinoma	negative
26	71	T ₂ N _{1b}	Anaplastic carcinoma	positive
27	72	T ₃ N ₂	Undifferentiated carcinoma	positive
28	67	T ₂ N _{1b}	Undifferentiated carcinoma	negative
29	83	T ₄ N ₂	Invasive ductal carcinoma	positive
30	63	T ₂ N _{1b}	Invasive ductal carcinoma	positive
31	61	T ₃ N ₀	Anaplastic carcinoma	negative
32	61	T ₃ N ₂	Invasive carcinoma	positive
33	49	T ₂ N ₀	Infiltrating carcinoma	negative

sulphate solution (21). Samples obtained at 9.30 hr were assayed and results were expressed in pmol/ml plasma.

Albumin was measured in all samples by a bromocresol green dye binding assay on the Technicon SMAC analyser using bovine serum albumin as standard.

Tumor measurements

At operation the specimen containing the tumor was placed on ice in the operating theatre and

immediately transferred to the laboratory. Following removal of sufficient material for histological examination, tumor was dissected free of fat and other tissues and divided into portions for assays of steroid receptor activity and steroid metabolism.

Steroid receptors

ER levels were determined by saturation analysis (22). A portion of tumor (300 mg) was homogenized (1:5 w/v) in 1 mmol/l Tris buffer containing monothioglycerol and centrifuged at 3200 rpm for

20 min. Aliquots of the resultant cytosol were incubated overnight at 4°C with 10 pg of (2, 4, 6, 7-³H) estradiol and varying amounts of competing non-radioactive estradiol (0, 0.03, 0.09, 0.15, 0.21, 0.28, 2.8 and 62 nmol/l). Bound steroid was separated from free by the addition of dextran-coated charcoal, and the bound fraction was measured after centrifugation by liquid scintillation counting. The concentration of receptors was determined by Scatchard analysis (23).

PR was assayed by a similar technique but using the synthetic progestogen promogestone (R5020) as ligand.

AR levels were assayed as above using the synthetic androgen methyltrienolone (R1881) after saturation of PR with promogestone (125 nmol/l). Using these conditions, less than 5% of the AR sites were blocked. Tumor cytosol protein (tcp) concentration was determined by the method of Bradford (24) and results for the three steroid receptors studied expressed as fmol/mg tcp. Values below 5 fmol/mg tcp for ER and 20 fmol/mg tcp for PR and AR were regarded as negative.

Steroid metabolism

Tumor (500 mg) was finely sliced and incubated for 2 hr at 37°C in Krebs Ringer phosphate buffer pH 7.4, containing an NADPH generating system and 22.5 μ Ci(7-³H) testosterone (8.9 Ci/mmol). The reaction was terminated by the addition of methanol, and the incubate was stored at -10°C until further processing. Before extraction of the metabolites, 500 μ g of non-radioactive carrier steroid was added for each metabolite to be purified so that procedural losses could be monitored. Metabolites were then separated and purified by thin layer chromatography and characterized by chemical derivative formation (25, 26). Metabolism was determined by measuring the percentage of radioactivity in the various characterized metabolites. Using these methods, conversions in excess of 0.02% were measurable. Values below this figure have been considered as negative.

Incubations performed in the absence of tumor showed no conversion to any of the metabolites investigated except Δ^4 androstenedione. This was less than 0.1%.

METABOLIC PATHWAYS STUDIED

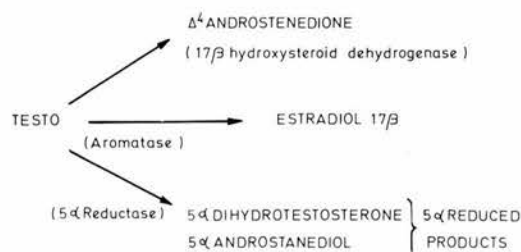


Fig. 1. Pathways of tumor testosterone metabolism investigated.

The pathways of steroid metabolism which were studied are shown in Fig. 1.

Statistical analysis

Because of the non-parametric distribution of these data, comparisons between groups were made by the Wilcoxon Rank Sum Test.

Results

Plasma hormones and hormone-binding proteins

Plasma prolactin concentrations in patients who were taking medication and those who were not are shown in Fig. 2. The mean level was significantly higher in those patients on drugs ($p < 0.02$), although one half of them still had values within the range displayed by the control patients.

The concentrations of the other plasma hormones and hormone-binding proteins are shown in Table 4. No significant differences were found between the two groups of patients.

Patients were regrouped on the basis of plasma prolactin concentrations to compare those with elevated prolactin levels (9 patients) with those within the control range (24 patients). Again no significant differences were found (Table 5).

Tumor steroid receptors

ER levels were measured in all 33 tumors, PR in 21, and AR in 19 (Fig. 3). No qualitative or quantitative differences in any of the three steroid recep-

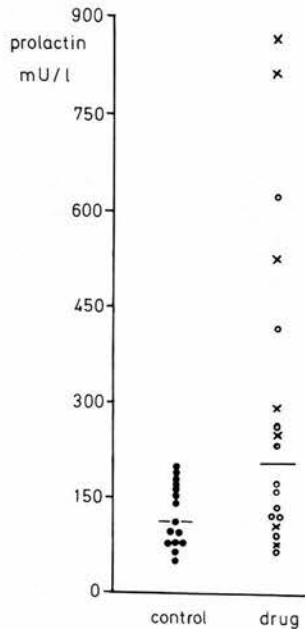


Fig. 2. Levels of plasma prolactin in controls and long-term drug patients (x: patients taking methyldopa; O: patients taking the other drugs studied). Prolactin levels were significantly greater in the drug patients ($p < 0.02$).

Table 4. Levels of plasma hormones and hormone-binding proteins in control and long-term drug patients.

	Control	Long-term drug	
Estrone	m 3.9	m 3.3	
ng/100 ml	r 1.5–5.5	r 0.6–6.5	$p > 0.1$
Estradiol	m 1.5	m 1.5	
ng/100 ml	r 1.0–2.1	r 0.7–2.2	$p > 0.1$
DHA	m 2.4	m 2.5	
ng/ml	r 0.2–5.2	r 1.5–7	$p > 0.1$
DHS	m 390	m 420	
ng/ml	r 70–1330	r 20–1760	$p > 0.1$
Testosterone	m 1.2	m 1.4	
nmol/l	r 0.6–2.4	r 0.3–3	$p > 0.1$
SHBG	m 73	m 70	
pmol/ml	r 32–178	r 15–195	$p > 0.1$
Albumin	m 44	m 43	
g/l	r 34–49	r 34–51	$p > 0.1$

m = median

r = range

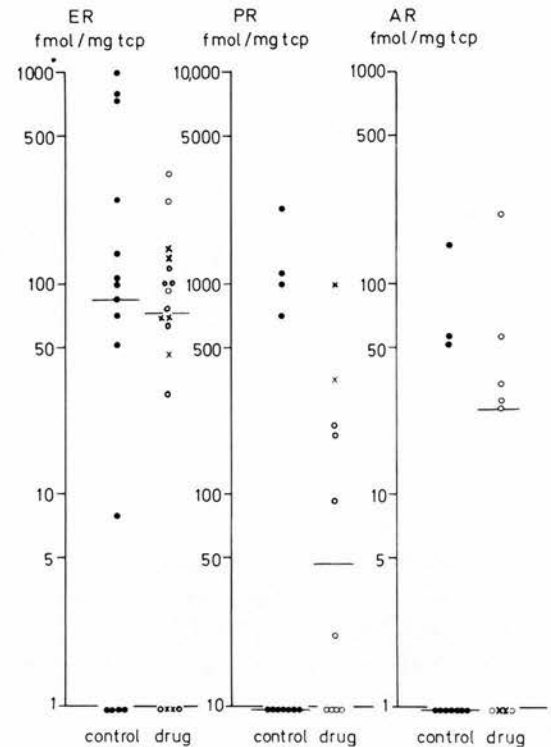


Fig. 3. Levels of tumor steroid receptors in controls and long-term drug patients (see Fig. 2 for symbols). No difference between these groups of patients for any receptor was significant ($p > 0.1$).

Table 5. Levels of plasma hormones and hormone-binding proteins in patients with normal and elevated levels of plasma prolactin.

	Plasma prolactin		
	Normal	Elevated	
Estrone	m 3.9	m 3.7	
ng/100 ml	r 1.4–6.5	r 0.6–4.4	$p > 0.1$
Estradiol	m 1.5	m 1.4	
ng/100 ml	r 0.8–2.2	r 0.7–2.2	$p > 0.1$
DHA	m 2.4	m 2.2	
ng/ml	r 0.2–6	r 1.0–7.0	$p > 0.1$
DHS	m 380	m 400	
ng/ml	r 70–1330	r 20–1760	$p > 0.1$
Testosterone	m 1.3	m 1.3	
nmol/l	r 0.6–3	r 0.3–2.1	$p > 0.1$
SHBG	m 72	m 68	
pmol/ml	r 32–178	r 15–195	$p > 0.1$
Albumin	m 43	m 41	
g/l	r 34–51	r 34–48	$p > 0.1$

m = median

r = range

tors were observed between the groups of patients ($p > 0.1$ in all cases). Regrouping of the data on ER on the basis of plasma prolactin also failed to show any significant differences.

Tumor steroid metabolism

The metabolism of testosterone and synthesis of androgens were measured in all cases. Twenty-one tumors were investigated for the synthesis of estrogen, of which 13 showed evidence of production (Figs. 4 and 5). There was a trend for decreased production of the three androgen metabolites studied in tumors from patients on medication, but this did not reach statistical significance.

After regrouping of the patients on the basis of plasma prolactin concentration there were still no significant differences in the amounts of testosterone metabolized or metabolites formed except for Δ^4 androstenedione (Fig. 6), which was signifi-

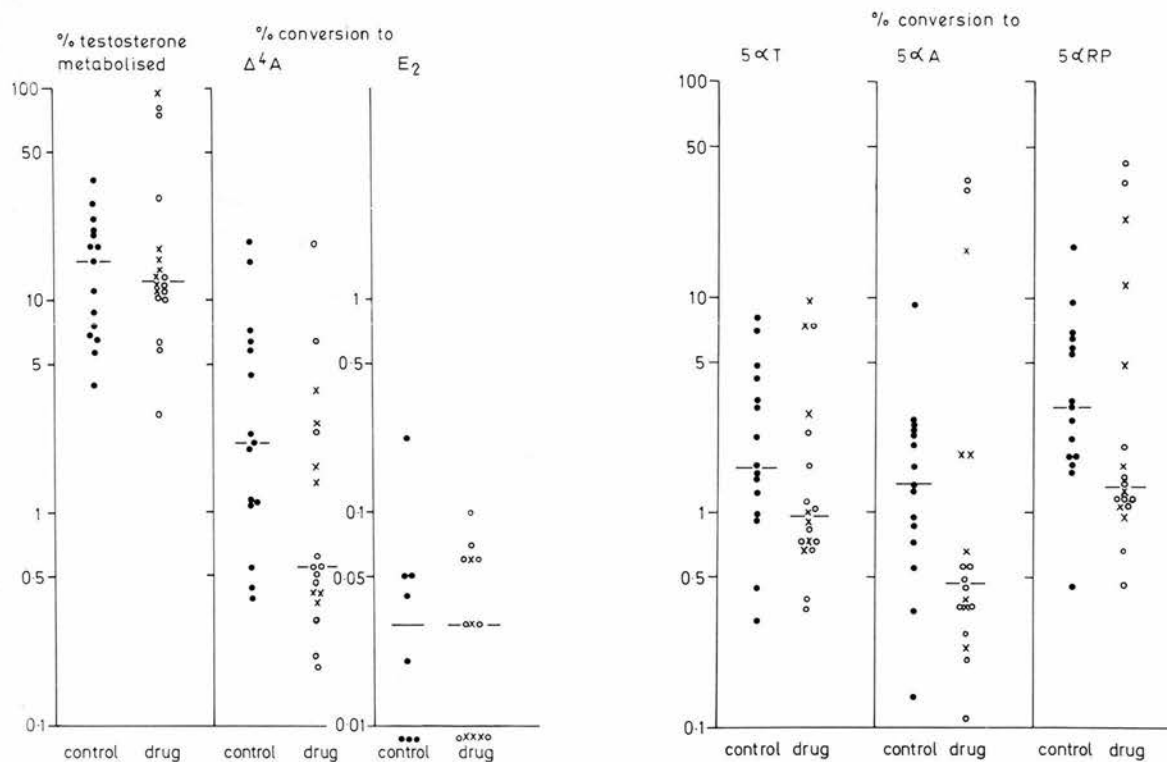
cantly decreased in the tumors of those patients with elevated prolactin ($p < 0.05$).

Methyldopa

Seven patients were taking this drug, which allowed separate statistical analyses to be performed comparing their results both with other patients receiving medication and with non-treated controls. No differences were found.

Discussion

An obvious problem in the design of this study was the multiplicity of drugs taken by patients. Only one drug subgroup (methyldopa) contained sufficient numbers to study separately. As this group did not differ from those remaining, it is valid to assume that the range of drugs studied had similar



Figs. 4 & 5. Levels of tumor testosterone metabolism in control or long-term drug patients (see Fig. 2 for symbols). No differences between these groups of patients for any facet of metabolism attained significance (Test = testosterone, Δ^4 A = Δ^4 androstenedione, E_2 = estradiol 17 β , $5\alpha T$ = 5α dihydrotestosterone, $5\alpha A$ = 5α androstenediol, $5\alpha RP$ = 5α reduced products $5\alpha T$ + $5\alpha A$).

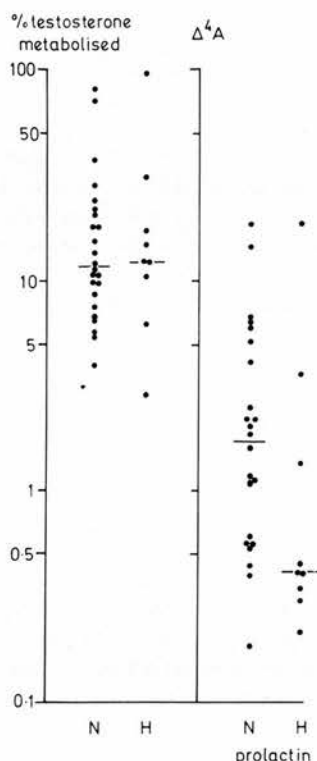


Fig. 6. Level of testosterone metabolism and conversion to Δ^4 androstenedione (Δ^4 A) in tumors from patients with normal (N) and elevated (H) plasma prolactin. The differences between the groups were insignificant for testosterone metabolism ($p > 0.1$) but significant for conversion to Δ^4 A ($p < 0.05$).

effects. A further complication in interpreting the results is that patients taking drugs were studied only at one time point, i.e. whilst on medication. As no data is available on the patients before the start of drug ingestion, it is not possible to state categorically that the drugs were completely without effect, despite the lack of difference from control patients. The likelihood that an earlier difference existed between the groups which was rectified by administration of the various medications seems highly improbable, especially as there was no common reason for prescribing the drugs.

It is of interest that the ingestion of these drugs was associated with elevated plasma prolactin concentrations in only half of the patients studied. We have reported (10) that the effect of phenothiazines on plasma prolactin is dose-related, but described a considerable individual variation between patients,

which has also been noted by others (27, 28). In the present study, ingestion of these drugs did not influence the levels of steroid hormones irrespective of the effect on plasma prolactin. This is contrary to the report of Vermeulen and Ando (15), who observed raised levels of adrenal androgens in hyperprolactinemic patients, but is in agreement with the findings of others (29, 30).

Our observation that these drugs also did not influence the levels of steroid receptors within the tumor or the ability of the tumor to metabolize steroids, even when hyperprolactinemia was induced, is in contrast to the results of our studies on rat DMBA-induced mammary tumors (13). However, these latter tumors are known to be particularly sensitive to prolactin.

It is therefore reasonable to assume that, in human breast cancer, events involving steroid hormones are not influenced by the drugs studied. In this respect we cannot present any evidence which would contra-indicate the use in breast cancer patients of drugs which coincidentally raise plasma prolactin levels.

Acknowledgement

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Androgen metabolism and apocrine differentiation in human breast cancer

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Keywords: androgen metabolism, apocrine differentiation, breast cancer

Summary

Metabolism of (7α - ^3H) testosterone has been measured in 111 human breast cancers and compared retrospectively with the degree of apocrine differentiation in the tumors. Cancers in which apocrine characteristics were a marked feature metabolized significantly more testosterone precursor than those in which apocrine features did not predominate. Higher metabolism was accounted for by increased conversion to 5α -reduced products such as 5α -dihydrotestosterone and 5α -androstanediols.

Introduction

Human breast cancers metabolize androgen precursors *in vitro* (1-3). Marked variations in level of metabolism occur between different tumors (4) but little is known about factors which influence such behaviour. A chance observation that a tumor which metabolized unusually large amounts of testosterone also had marked apocrine morphology on histological examination led to the present study, in which we have retrospectively related *in vitro* androgen metabolism to degree of apocrine differentiation.

Materials and methods

Tumors

Histologically proven cancers of the breast were

obtained at mastectomy from 111 patients. Following excision, tumors were immediately placed on ice. Sufficient tissue was removed for histopathological investigation including apocrine grading, and the remainder was used to determine the metabolism of testosterone.

Testosterone metabolism

A 500 mg portion of each tumor was finely sliced and incubated for 2h at 37°C in Krebs Ringer phosphate buffer pH 7.4 containing a NADPH generating system and 22.5uCi 7α - ^3H testosterone. The reaction was stopped by addition of methanol (40 ml) and the incubations were stored at -10°C until being processed further. Before extraction of metabolites, 500 ug of non-radioactive carrier of the steroids to be characterized were added to enable monitoring of procedural losses. Metabolites were then extracted as described by Fahmy et al. (5) and

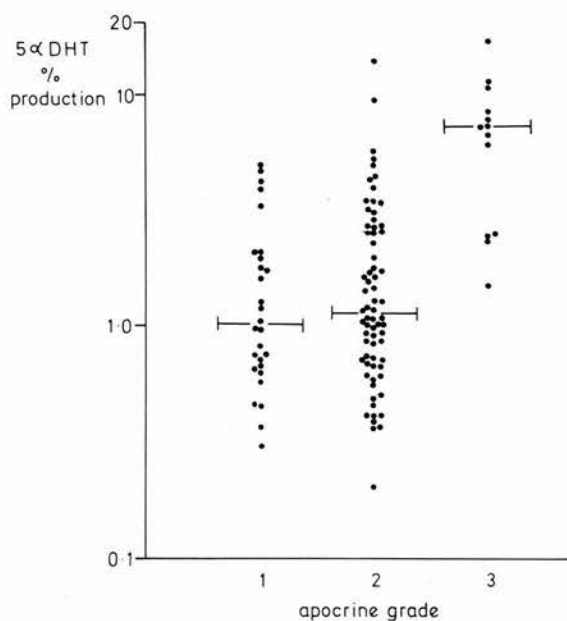


Fig. 2. Percentage conversion of testosterone to 5 α -dihydrotestosterone by human breast cancers subdivided according to grade of apocrine differentiation (see Methods). Horizontal lines refer to median value. There is no significant difference between grades 1 and 2, but grade 3 is significantly different from both ($p < 0.01$ by Wilcoxon Rank Test).

graded with 5 α -androstane-17 β -diol. This behaviour is consistent with the original component being epiandrosterone and/or androsterone.

Discussion

All human breast cancers metabolize testosterone *in vitro*, but large quantitative variations in metabolism occur between different tumors and little is known of factors which influence activity. In the present study, the association between degree of tumor apocrine differentiation and metabolic potential has been investigated.

It has long been recognised that human breast cancers may show apocrine features (7–17). The incidence of apocrine differentiation varies in reported series according to the criteria used by different authors to define apocrine characteristics. Carcinomas with marked apocrine features are most easily recognised by the characteristic copious pale eosinophilic cytoplasm (13, 14), although care

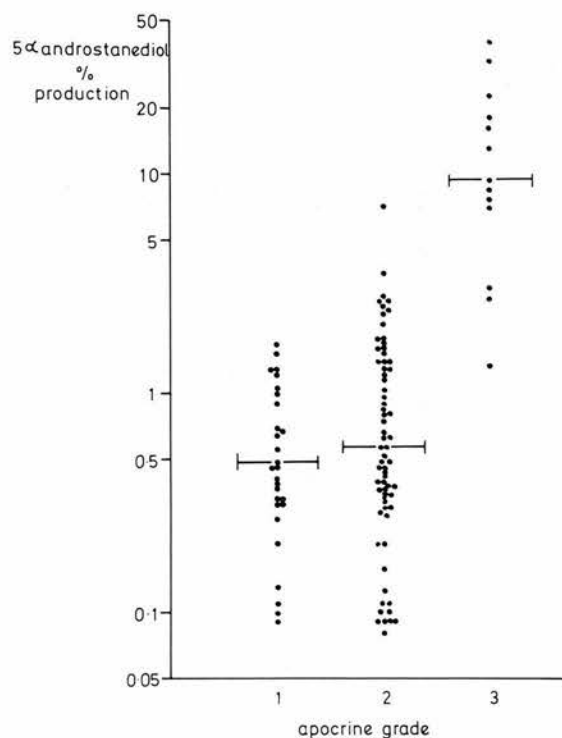


Fig. 3. Percentage conversion of testosterone to 5 α -androstane-17 β -diol by human breast cancers subdivided according to grade of apocrine differentiation (see Methods). Horizontal lines refer to median value. There is no significant difference between grades 1 and 2, but grade 3 is significantly different from both ($p < 0.01$ by Wilcoxon Rank Test).

must be taken not to confuse this with staining artefact. Secretory snouts and vesicular nuclei with prominent nucleoli, both features of benign apocrine epithelium, may also be seen (15). Using these criteria it is generally accepted that between 2 and 15% of all breast carcinomas show marked apocrine differentiation (10, 13, 17), although up to 62% may show apocrine characteristics without this being a striking feature of the tumor (15). The finding in this series of 12% with marked and 63% with some degree of apocrine differentiation, the remainder having minimal apocrine features, therefore correlates well with these previous studies.

With regard to metabolism of testosterone, large variations were observed between tumors within the groups categorized as having either minimal or moderate apocrine features, and there was no sig-

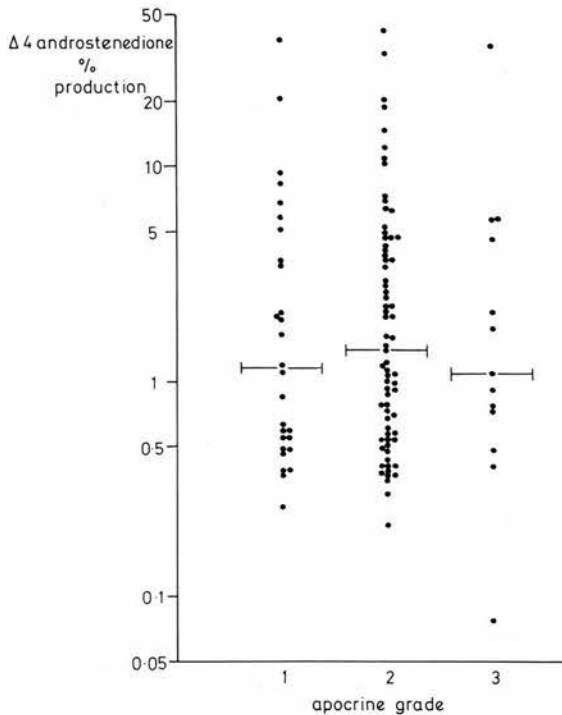


Fig. 4. Percentage conversion of testosterone to Δ^4 -androstenedione by human breast cancers subdivided according to grade of apocrine differentiation. Horizontal lines refer to median value. There are no significant differences between the groups by the Wilcoxon Rank Test.

nificant difference in any aspect of metabolism between these two groups. This would indicate that in tumors where apocrine development is not a predominant feature, the degree of development does not account for variability in testosterone metabolism. However, tumors with marked apocrine features metabolized significantly greater amounts of testosterone than the groups of tumors with lesser apocrine differentiation. Increased metabolism was associated with raised production of 5α -dihydrotestosterone and 5α -androstanediols. Data from scans of the distribution of radioactivity in metabolites also suggested increased transformation to other 5α -reduced steroids such as 5α -androstanedione, androsterone, and/or epiandrosterone. In contrast, conversion to Δ^4 -androstenedione was not enhanced. It therefore seems unlikely that raised metabolism in tumors with markedly apocrine features is due to a non-specific effect such as a general increase in cellularity.

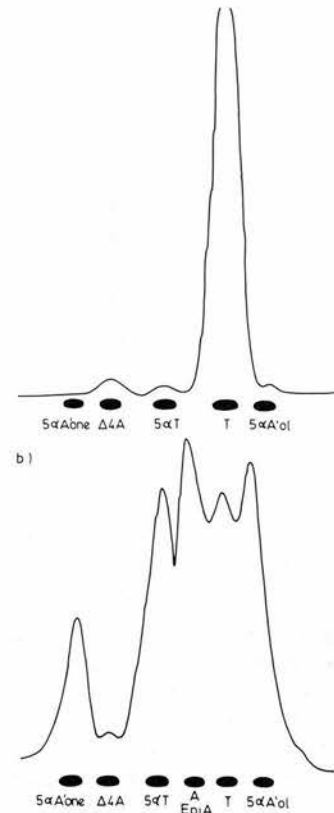


Fig. 5. Typical scans of radioactivity on the t.l.c. plates after initial chromatography: (a) from a tumor with minimal apocrine differentiation (Grade 1), and (b) from a tumor with a marked apocrine differentiation (Grade 3). Spots corresponded to areas in which radioinert steroid standards migrated. $5\alpha A'one$ = 5α -androstanedione; $\Delta^4 A$ = Δ^4 -androstenedione; $5\alpha T$ = 5α -dihydrotestosterone; A = androsterone; $EpiA$ = epiandrosterone; T = testosterone; $5\alpha A'ol$ = 5α -androstanediol.)

Several tumors with marked apocrine features were incubated as explants in short-term organ culture with 7α - 3H testosterone. These all showed enhanced 5α -reduction compared with carcinomas without such differentiation. This would suggest that tumors with marked apocrine features inherently possess high 5α -reduction and that the activity observed in the present study is not artefactual of the specific incubation conditions or co-factors employed.

Variations in testosterone metabolism have been reported to occur between samples of skin taken from different areas of the body (18) and it is thus relevant that axillary and perineal skin which con-

tain cells from apocrine sweat glands display high metabolism to 5 α -reduced steroids (18, 19). This activity of apocrine tissue probably accounts for the high concentrations of 5 α -reduced androgens which are found in apocrine fluids (20). It is also of interest that in human breast cancers the production of GCDFP-15, a protein marker of apocrine activity (16), may be stimulated by administration of androgens (21), and that in rabbits androgens stimulate the growth and activity of normal apocrine glands (22).

It remains to determine whether raised 5 α -reductase in breast cancers with marked apocrine features is of biological or clinical significance. 5 α -dihydrotestosterone retards the growth of rat mammary tumors (23). It also inhibits estrogen synthesis in breast cancer cells (24) and competes with estradiol for estrogen receptors (25). Tumors with high 5 α -reductase activity might therefore be expected to be associated with good prognosis; as yet, the present data has not been examined to determine if this is so. Although some workers have suggested that apocrine cancers have a better prognosis (26), others have failed to show that they have a different prognosis from other histological types of breast cancer (8, 11).

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Chapter 12

Steroid Metabolism in Breast Cancer

W. R. MILLER

Steroid hormones which may influence the growth and development of both normal and diseased breast are classically synthesized from cholesterol (Figure 12.1). At one time, such biosynthesis was thought to be exclusive to the gonads, adrenal cortex and fetal-placental unit, but it is now recognized that other organs, including breast and its tumours, perform many of the transformations. Such potential may be important in influencing the steroid environment within the breast and in the vicinity of breast tumours.

The evidence for the existence of such steroid metabolism will be reviewed for breast cancer, benign breast disease, normal breast tissue and breast fat.

STEROIDOGENESIS IN BREAST CANCER

While the evidence that breast cancers can synthesize active hormones from cholesterol is unconvincing (Abul Hajj 1984; Duncan *et al.* 1981), there is no doubt that they can use partially formed precursors which have been produced elsewhere and released into the circulation. These 'pro hormones' include androgens such as dehydroepiandrosterone (DHA) sulphate, which are produced by the adrenal (Abraham & Chakmakjian 1973) and may accumulate in the breast in high concentrations (Miller & Forrest 1983). Results from studies involving *in vitro* incubation (Adams and Wong 1968; Cameron *et al.* 1971; Couch *et al.* 1975) or perfusion of the breast *in situ* (Jones *et al.* 1970) indicate that most, if not all, breast cancers metabolize DHA sulphate to other androgens such as DHA itself, $\Delta 4$ androstenedione and testosterone. These metabolites are, in turn, the more immediate precursors of active hormones.

Breast cancers, for example, have the capacity to convert testosterone into more potent androgens such as 5α dihydrotestosterone and 5α androstane diols (Jenkins & Ash 1972, McIndoe & Woods 1981; Miller *et al.* 1973). Such 5α reductase activity is classically associated with the prostate in which 5α reduced products promote the development and function of the gland (Mainwaring 1977). All breast cancers have capacity for 5α reduction but the level varies greatly between different tumours (Miller *et al.* 1978), while rarely reaching that displayed by the prostate (Perel & Killinger 1983).

Testosterone and $\Delta 4$ androstenedione are the immediate precursors of oestrogen

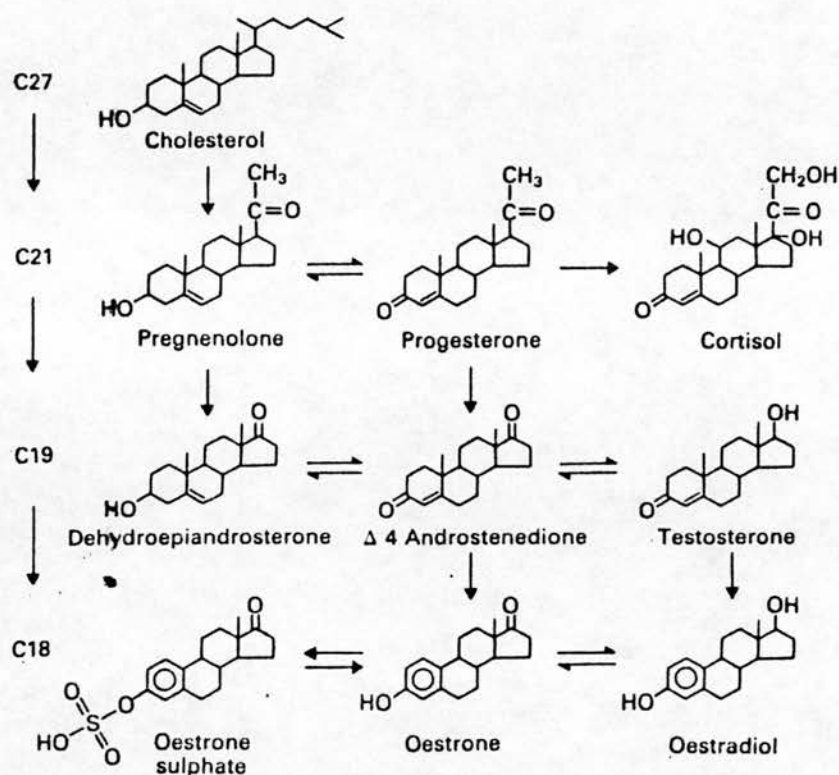


Figure 12.1 Major pathways of steroid hormone biosynthesis.

(Figure 12.1) and several groups of workers have provided unequivocal evidence for the conversion of androgens into oestrogens or 'aromatization' by human breast cancer (Table 12.1). The proportion of cancers which have this potential varies from around 50 per cent to 100 per cent in different reported series. Using a method of rigorous characterization we have obtained evidence of oestrogen production in just over one-half of breast cancers (Miller *et al.* 1982a). The levels of oestrogen synthesized are small in comparison with those in classical endocrine organs, such as the ovary and placenta, but are comparable to, or higher than, levels in other peripheral organs with oestrogen biosynthetic activity (Abul-Hajj *et al.* 1979a; Perel *et al.* 1980).

Because other cells, such as adipocytes, may also produce oestrogen (see below), it is important to demonstrate that oestrogen biosynthesis in specimens of tumour is truly associated with cancer cells. In this respect, studies of cell lines of breast cancers are of value and, despite an early negative finding (D'Agata *et al.* 1978), it is clear that under optimal culture conditions breast-tumour cells will synthesize oestrogen from androgen precursors (MacIndoe 1979). Furthermore, different clones of malignant

Table 12.1 Oestrogen biosynthesis from androgen precursors in human breast cancers incubated *in vitro*.

Androgen precursor	Oestrogen product	Incidence	References
DHA	Oestrone	7/18 (39%)	Abul-Hajj <i>et al.</i> (1979b)
		2/12 (17%)	Miller W.R. (unpublished)
$\Delta 4$ androstenedione	Oestradiol	11/18 (61%)	Abul-Hajj <i>et al.</i> (1979b)
	Oestrone	17/17 (100%)	Perel <i>et al.</i> (1981)
		7/17 (41%)	Varela & Dao (1978)
	Oestradiol	17/17 (100%)	Perel <i>et al.</i> (1981)
		10/17 (59%)	Varela & Dao <i>et al.</i> (1978)
Testosterone		4/6 (67%)	Miller W.R. (unpublished)
	Oestradiol	9/15 (60%)	Abul-Hajj <i>et al.</i> (1979b)
		21/23 (91%)	Li & Adams (1981)
		66/110 (60%)	Miller <i>et al.</i> (1982a)

cells from the same tumour may display differing potential for oestrogen production, thereby demonstrating heterogeneity of aromatase activity in cells from a single tumour (Perel *et al.* 1983).

Breast cancers also have the capacity to interconvert oestrogens (Figure 12.1). Oestrone and its sulphate represent the major, although relatively inactive, circulating oestrogens in postmenopausal women (Baird *et al.* 1969a; Samojlik *et al.* 1982) but both may be metabolized into the more potent hormone, oestradiol (Vignon *et al.* 1980; Wilking *et al.* 1980; Willcox & Thomas, 1972). Equally, however many breast cancers can inactivate oestradiol by performing the reverse conversions to oestrone (Abul-Hajj *et al.* 1979b; Geier *et al.* 1975; Willcox & Thomas 1972) or oestrogen conjugates (Braunsberg *et al.* 1974. Dao & Libby 1972; Leung *et al.* 1973). The balance of these oestrogen interconversions might be critical in determining the net oestrogenicity within the tumour.

STEROIDOGENESIS IN BENIGN BREAST DISEASE

Benign breast disease encompasses a variety of lesions. While there is no evidence that any type of benign lesion can transform cholesterol into steroid hormones, interconversions of androgen and oestrogen, such as those described above, occur also in several types of benign lesions (Geier *et al.* 1974; Jenkins & Ash 1972; Lloyd 1979; Miller *et al.* 1973; Perel *et al.* 1981; Willcox & Thomas 1972). In general, the transformations occur to a lesser extent than in malignant tissue, with the exception of 5α reduction in cystic disease and certain fibroadenomas, which may display high levels of activity (Lloyd 1979; Miller *et al.* 1973).

High levels of steroid hormones may be found in fluids aspirated from breast cysts (Bradlow *et al.* 1981; Miller *et al.* 1982b) and, for example, the median value of DHA sulphate is on average 50-fold higher than that in plasma (Miller *et al.* 1983a).

As high levels of oestrogen conjugates are also present in cyst fluid but are not concentrated from plasma, it has been inferred that these may be synthesized locally from androgen precursors (Raju *et al.* 1984).

It has recently been shown that breast carcinomas with marked apocrine differentiation show enhanced ability to metabolize testosterone to 5 α -reduced products such as 5 α -dihydrotestosterone and 5 α -androstane-3 β -diols (Miller *et al.* 1984). This observation may explain why cystic lesions of the breast which are frequently associated with apocrine activity (Haagensen *et al.* 1981) should also display a high capacity for 5 α reduction.

STEROIDOGENESIS IN NORMAL BREAST TISSUE

Specimens of normal breast tissue taken from areas of mastectomy specimens far removed from any malignancy show evidence of steroidogenic potential (Couch *et al.* 1975; Geier *et al.* 1974, 1975; Lloyd 1979; Miller *et al.* 1973; Perel *et al.* 1981). Metabolism of progestogens, androgens and oestrogens have all been reported, including the pathways discussed above. The levels of conversions in normal breast are generally lower than those in benign or malignant breast tissues, and this may be due in part to the low cellularity of 'normal' resting breast.

Considerable attention has been given to the possibility that normal breast may synthesize oestrogens but most workers have not detected steroid aromatizing potential in normal breast tissue (Abul-Hajj *et al.* 1979a; Deshpande *et al.* 1976; Miller *et al.* 1978). The single group reporting oestrogen biosynthesis described activities which were much lower than those found in 'parallel' incubations of malignant tissue (Perel *et al.* 1980). The general inability to detect oestrogen biosynthesis in normal breast may be due to its low cellularity. Studies of cell lines in culture should overcome this problem and, recently, biosynthesis has been detected in epithelial cells derived from human milk (Perel *et al.* 1984a).

STEROIDOGENESIS IN BREAST FAT

It is clear that fat has significant, albeit limited, capacity for steroid interconversions (Bleau *et al.* 1974; Perel & Killinger 1979). Among the transformations validated are those of DHA to Δ 5 androstenediol (Schindler & Aymer 1973), Δ 4 androstenedione to testosterone (Perel & Killinger 1979), aromatization of androgens to oestrogens (Bolt & Gobel 1972; Schindler *et al.* 1972) and the interconversion of oestrone and oestradiol (Bleau *et al.* 1974).

In postmenopausal women, aromatization in peripherally distributed fat probably represents one of the major sources of circulating oestrogen (Grodin *et al.* 1973) and there are positive correlations between body weight, degree of obesity and levels of plasma oestrogens (MacDonald *et al.* 1978; Vermeulen & Verdonck 1978). Samples of fat from various parts of the body including breast have been shown to

synthesize oestrogen *in vitro* (Beranek *et al.* 1984; Perel & Killinger 1979). In view of the relative increase in the proportion of fat to parenchyma in the breast after the menopause (Anastassiades *et al.* 1983), local production of oestrogen by breast fat may be particularly important in postmenopausal women.

CONTROL OF STEROID METABOLISM IN THE BREAST

While pituitary hormones such as growth hormone and prolactin may be trophic for the breast (Dilley & Kister 1975; Peters *et al.* 1983; Shiu 1982) there is not any good evidence that they regulate steroid interconversions in the breast. Although hyperprolactinaemia is associated with enhanced testosterone metabolism in carcinogen-induced rat mammary tumours (Miller 1976), these tumours have a particular sensitivity to prolactin (Meites 1972; Pearson *et al.* 1972) which is rarely seen in human breast cancers (Legha & Swenerton 1979). Drug-induced hyperprolactinaemia in patients with breast cancer is not associated with differences in steroid metabolism, either by normal or malignant breast (Mason *et al.* 1983).

Steroid metabolism in the breast may nevertheless, be controlled by polypeptide hormones. Aromatization of androgens to oestrogen by human breast cancer is influenced by cyclic AMP (Miller unpublished) and factors which control the production of cyclic AMP in the breast are therefore likely to affect oestrogen biosynthesis. The situation is analogous to that in adipose tissue where cyclic AMP stimulates oestrogen formation (Simpson *et al.* 1983).

Metabolism of steroid hormones is also modulated by steroids themselves (reviewed by Gower & Cooke 1983). For example, progesterone appears to increase 17 β hydroxysteroid dehydrogenase activity not only in the endometrium (Buirchell & Hähnel 1975; Tseng & Gurpide 1975) but also in breast fibroadenomas (Fournier *et al.* 1982). This would account for the maximum enzyme activity found in normal breast tissue during the early secretory phase of the menstrual cycle (Pollow *et al.* 1977). *In vitro* studies show that oestrogen inhibits 5 α reduction (Miller & Forrest 1978) and, conversely, 5 α reduced androgens block oestrogen biosynthesis in human breast cancer (Perel *et al.* 1984b).

A marked induction of aromatase activity occurs in cultures of cells from either breast cancer or adipose tissue in the presence of cortisol and dexamethasone (Folkerd & James 1983; Perel *et al.* 1983; Simpson *et al.* 1983). Prolactin may be synergistic in this system (Folkerd & James 1984). Whether such hormonal effects apply only to culture conditions or are reproduced also *in vivo* is not known.

RELATIONSHIP TO STEROID RECEPTORS

There has been considerable interest in the possible association between tumour steroid metabolism and the presence of hormone receptors (Table 12.2). Particular attention has been given to the relationship between oestrogen biosynthesis and

Table 12.2 Relationship between steroid metabolism and steroid receptors in breast cancer. ER = oestrogen receptor; E₂ = oestradiol 17 β ; PgR = progesterone receptor; AR = androgen receptor.

Steroid conversion	Reference	Tumours	Relationship
Oestrogen biosynthesis	Abul-Hajj <i>et al.</i> (1979a)	18	Incidence lower in ER + ve cf. ER - ve.
	Li & Adams (1981)	23	Incidence similar in ER + ve and ER - ve. No correlation between level of E ₂ synthesized and ER status.
	Miller <i>et al.</i> (1979)	40	Incidence not significantly different in ER + ve and ER - ve.
	Miller <i>et al.</i> (1982a)	110	Incidence higher in ER + ve cf. - ve. Levels of synthesis not correlated with ER status or level. Level of ER higher in tumours with E ₂ synthesis.
	Tilson-Mallet <i>et al.</i> (1983)	61	Level of synthesis similar in ER + ve and ER - ve. No correlation with PgR status.
	Varela & Dao (1978)	17	Incidence not correlated with ER status.
Oestrogen 17 β dehydrogenase	Abul-Hajj <i>et al.</i> (1979b)	31	Conversion higher in ER poor cf. ER rich.
	Wilking <i>et al.</i> (1980)	23	Conversion higher in ER poor cf. ER rich.
Oestrogen sulphotransferase	Godfroi <i>et al.</i> (1975)	20	Positive correlation with ER status.
	Leung <i>et al.</i> (1973)	26	Positive correlation with ER status in primary cancer.
	Pewnin <i>et al.</i> (1982)	62	Activity higher in ER + ve cf. ER - ve.
	Pewnin <i>et al.</i> (1980)	44	Activity higher in PgR + ve cf. PgR - ve.
	Tseng <i>et al.</i> (1983)	66	Activity higher in ER + ve/PgR + ve cf. ER - ve/PgR - ve.
Oestrogen sulphatase	Santner <i>et al.</i> (1984)	35	No correlation with ER status.
	Tseng <i>et al.</i> (1983)	66	No correlation with ER status.
	Wilking <i>et al.</i> (1980)	23	No correlation with ER status.
5 α reduction	Abul-Hajj (1979)	16	Activity higher in ER + ve cf. ER - ve.
	Mason <i>et al.</i> (1981)	79	Activity lower in ER + ve cf. ER - ve.
	Mason (1981)	30	No correlation with AR status.

oestrogen receptors. It is clear that oestrogen production may be demonstrated in both receptor-positive and -negative tumours (Li & Adams 1981; Miller *et al.* 1982a). There is, however, no consensus of opinion about whether oestrogen-receptor-positive tumours are more or less likely to produce oestrogen than their receptor-negative counterparts.

Several factors may account for disparity between results; these include different cut-off values for defining oestrogen-receptor-positive tumours, different substrates

and methods for assessing tumour aromatase, and the variation in detection limits associated with different characterization techniques for oestrogen. Our own studies showed a significant positive correlation when larger numbers of tumours were examined (Miller *et al.* 1982a) and within the group of oestrogen-receptor-positive tumours, the level of receptor was significantly higher in cancers producing oestrogen compared to those without biosynthetic activity. However, the level of oestrogen synthesis does not apparently differ between oestrogen-receptor-positive and -negative tumours (Li & Adams 1981; Miller *et al.* 1982a; Tilson-Mallet *et al.* 1983).

Other aspects of tumour oestrogen metabolism have been reported to be significantly related to the presence of oestrogen receptors. Thus, there are higher levels of the oestradiol 17 β dehydrogenase enzyme converting oestradiol into oestrone in oestrogen-receptor-poor tumours, compared with those which are receptor-rich (Abul-Hajj *et al.* 1979b). Several groups have shown that there is also a positive correlation between the ability of tumours to sulphate oestrogen and the presence of oestrogen receptors (Table 12.2).

Conflicting data have emerged over the relationship between oestrogen receptors and the production of 5 α reduced androgens. In a small number of tumours, Abul-Hajj (1979) reported a positive correlation between these parameters, whereas our investigations on a larger series of cancers derived from postmenopausal patients alone, showed higher 5 α reductase activity in oestrogen-receptor-negative tumours (Mason *et al.* 1981). No significant relationship has been detected between tumour androgen-receptor level and the production of its natural binding steroid, 5 α dihydrotestosterone (Mason 1981).

BIOLOGICAL SIGNIFICANCE OF STEROIDOGENESIS IN BREAST CANCER

If steroid metabolism within the breast is of biological importance, its role must be local within the breast itself. Effects at sites distant from the breast are improbable because of the relatively low levels of steroid interconversions within breast tissues and because, unlike the case of lung tumours (Bower and Gordon 1965), widespread responses attributable to inappropriate hormone production have not been reported in patients with breast cancer.

Local hormone biosynthesis may not be important in premenopausal breast cancer where the ovary releases sufficient steroid hormones into the circulation to account for the maintenance of hormone-dependent neoplastic growth. It may be more important in premenopausal cystic disease in which high levels of steroid conjugates are present within cyst fluid (Bradlow *et al.* 1981; Miller *et al.* 1982a). Even a small conversion of these inactive precursors into more active products might be associated with marked changes in the hormonal milieu within the breast.

However, in the postmenopausal woman, steroid metabolism by the breast may have relevance to breast cancers which require steroid hormones for their continued growth. After the menopause, the ovary ceases oestrogen and progestogen

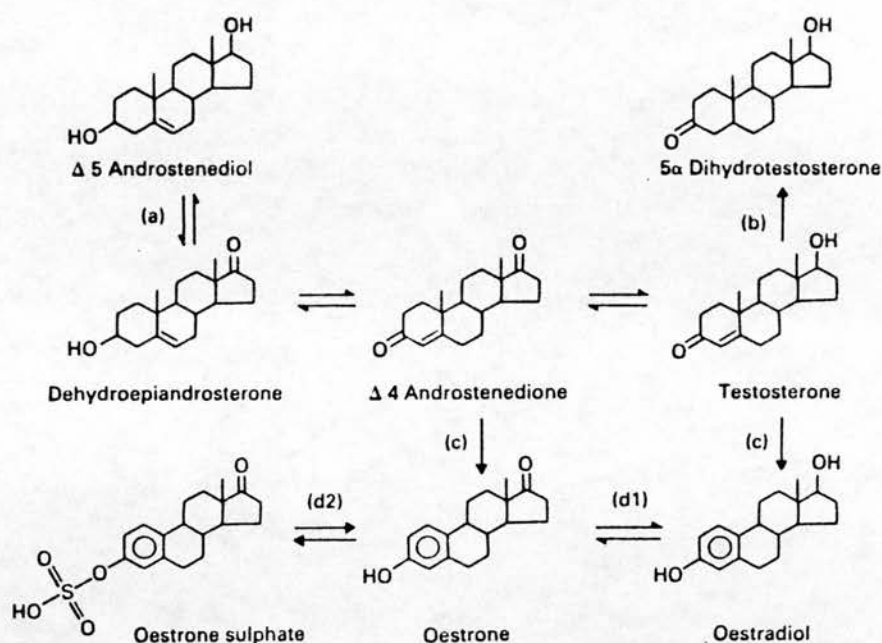


Figure 12.2 Metabolic conversions producing major changes in biological activity of steroids: (a) formation of the oestrogenic androgen, $\Delta 5$ androstenediol; (b) synthesis of the potent androgen, 5 α dihydrotestosterone; (c) aromatization of androgens to oestrogens; and (d) interconversion of oestrogens either by 17 β dehydrogenase (d1) or sulphokinase sulphatase (d2).

production, although it still secretes some androgen (Vermeulen 1976). The adrenal cortex also synthesizes little oestrogen (Baird *et al.* 1969b) and, while it produces large amounts of androgens such as androstenedione, dehydroepiandrosterone and its sulphate (Baird *et al.* 1969b; Nieschlag *et al.* 1973), these steroids appear to be largely inactive within the breast. Nevertheless, such androgens may be metabolised at peripheral sites into hormones which do have biological effects on breast cells. These peripheral sites include adipose tissue (Bleau *et al.* 1974; Perel & Killinger 1979), liver (Smuk & Schwiers 1977), skin (Schweikert *et al.* 1976), muscle (Longcope *et al.* 1978) and, as already discussed, the breast itself. The following discussion reviews the possible biological significance in breast cancer of specific aspects of steroid metabolism (Figure 12.2).

Synthesis of $\Delta 5$ androstenediol

The biological importance of $\Delta 5$ -androstenediol resides in its oestrogenic activity (Adams 1983). The steroid binds to oestrogen receptors (Thijssen *et al.* 1975) and is capable of eliciting oestrogen-mediated responses, for example induction of progesterone receptors (Kreitman & Bayard 1979). Concentrations of $\Delta 5$ -androstenediol required for these effects are about 2nM (Adams *et al.* 1981), which is about 40-fold

higher than the level of oestradiol necessary for the same response. Thus, where oestradiol levels are low in comparison with androgens, as for example in postmenopausal women, $\Delta 5$ androstenediol may act as an oestrogen, or at least supplement the stimulus of natural oestrogens.

Measurements of relative levels of $\Delta 5$ androstenediol in tumours (Adams *et al.* 1980; Maynard *et al.* 1978; Van Landeghem 1984) support the concept that the steroid has an oestrogenic potential, particularly in cancers from postmenopausal women. It remains to be determined whether the tumour concentrations of $\Delta 5$ androstenediol result from local synthesis. Breast cancers have the capacity to convert DHA and its sulphate to $\Delta 5$ androstenediol (Adams 1983; Couch *et al.* 1975) and there are large amounts of these precursors in the breast (Miller *et al.* 1980). Furthermore, as both free and conjugated DHA have very little oestrogenic activity (Adams *et al.* 1981) their conversion to $\Delta 5$ androstenediol would necessarily increase the oestrogenic environment of the tumour.

5 α -reduction

5 α -reduced androgens are among the most potent androgenic steroids and, indeed, 5 α dihydrotestosterone forms the natural ligand for androgen receptors (for review see Liao 1977). Tissues which are sensitive to androgens invariably show high 5 α -reductase activity (Wilson 1972). It is therefore of interest that breast tissues may also show a high potential for 5 α -reduction (Miller *et al.* 1978) and that about 30–40 per cent of breast cancers possess cytoplasmic androgen receptors (Miller *et al.* 1983b; Persijn *et al.* 1975; Teuling *et al.* 1980). However, it has not been possible to show any correlation between tumour 5 α -reduction and the presence of androgen receptors (Mason 1981) and despite a recent report (Hackenberg *et al.* 1984) anti-androgen therapy has produced little impact on the treatment of breast cancer.

If, therefore, tumour 5 α -reductase activity is of biological significance, it is likely to be independent of androgen receptors. For example, since 5 α -reduced steroids are powerful inhibitors of aromatase activity (Gower & Cooke 1983), tumour 5 α -reductase activity might control oestrogen biosynthesis *in situ*. In keeping with this, an inverse correlation has been reported between 5 α -reduction and aromatase activity in breast-cancer cells (Perel *et al.* 1984b). Some synthetic androgens which have been used successfully in the treatment of advanced breast cancer have also subsequently proved to be aromatase inhibitors (Dao 1982).

Aromatase

Amounts of oestrogen synthesised from added substrate during *in vitro* incubations of breast cancers are sufficient to elicit oestrogen-mediated events (Miller *et al.* 1982a). However, calculations on the concentrations of androgen precursors present as endogenous substrate in the tissue suggest that tumour aromatase is likely to produce sufficient oestrogen for meaningful responses in only about ten per cent of all breast cancers (Bradlow 1982; Tilson-Mallett *et al.* 1983). Although this may

represent only a small number of tumours, it could be significant if these were restricted to the one-third of cancers which respond to oestrogen-deprivation therapy.

A critical factor in evaluating the significance of aromatase activity is the amount of available androgen precursors. As the breast appears to concentrate certain of these compounds (Bradlow *et al.* 1983), calculations based on the levels of circulating androgen may be misleading. To date, little account has been taken of the high concentrations of androgen conjugates which may be present in the breast (Miller & Forrest 1983). In view of the existence of sulphatase activity within the breast (Adams & Wong 1968), the pool of these precursors ought not to be overlooked in terms of either being directly converted to oestrogen or acting as a reservoir to supplement the flux of free androgen substrate.

Theoretical calculations indicate that tumour aromatase activity is insufficient to account for the levels of endogenous oestrogen within the tumour (Bradlow 1982; Tilson-Mallet *et al.* 1983), which in postmenopausal women may be greatly in excess of concentrations in the plasma (Edery *et al.* 1981; Fishman *et al.* 1977; Millington *et al.* 1974). However, it is equally difficult to account for these high endogenous levels of oestrogen by other means. Tumour-oestrogen concentrations do not reflect levels in peripheral plasma (Nagai *et al.* 1979; Vallent *et al.* 1982). Nor has any reproducible correlation been found between endogenous oestrogens and levels of oestrogen receptors (Edery *et al.* 1981; Nagai *et al.* 1979; Van Landeghem *et al.* 1985), the mechanism by which oestrogen would be most likely trapped within tumour cells.

The presence of aromatase activity in both oestrogen-positive and -negative tumours and the lack of any relationship between aromatase and progesterone receptor status (Table 12.2) does not necessarily argue against tumour oestrogen biosynthesis being of physiological significance. Aromatase activity may have a biological role independent of receptors or may be of significance only in steroid receptor-positive tumours. Abul-Hajj (1975) has advocated the former possibility suggesting that oestrogen-receptor-negative tumours appear endocrine independent only because they can satisfy their hormone needs by means of their own biosynthetic capacity. This seems unlikely because tumours responding to therapy with aromatase inhibitors or antioestrogens (which ought to be effective irrespective of the source of oestrogen) are invariably oestrogen-receptor-positive (Hawkins *et al.* 1980; Lawrence *et al.* 1980). It seems more likely that if oestrogen biosynthesis is to be of significance, it will be in oestrogen-receptor-positive tumours where a mechanism exists to process the synthesized steroid. Indeed, if *in situ* synthesis is a major route by which certain breast cancers obtain their oestrogen, then it might be expected that these tumours will respond to therapies which inhibit tumour aromatase.

A study has been set up in which both tumour oestrogen receptors and potential for aromatization are measured in oestrogen-receptor-positive tumours from postmenopausal women with advanced breast cancer who are to be subsequently treated with aminoglutethimide, an aromatase inhibitor. The numbers of patients studied are, as yet, small but preliminary data suggest that tumours with both high aromatase activity and oestrogen receptors are most likely to respond (Table 12.3). Screening for tumour aromatase may therefore select those most likely to respond to antiaromatase

Table 12.3 Relationship between tumour aromatase and response to aminoglutethimide in patients with advanced breast cancer. All tumours were oestrogen-receptor-positive (exceeding 5 fmol mg⁻¹ cytosol protein).

Patient	Tumour aromatase* (%)	Clinical Response
1	0.18	Partial regression
2	0.09	Partial regression
3	0.08	Partial regression
4	0.08	Partial regression
5	0.08	Progression
6	0.06	Partial regression
7	0.06	Progression
8	0.04	Partial regression
9	0.04	Progression
10	0.03	Partial regression
11	0.03	Partial regression
12	<0.02	Progression
13	<0.02	Progression
14	<0.02	Progression
15	<0.02	Progression

*Figures refer to percentage production of oestradiol from [³H] testosterone by the method described by Miller & Forrest (1974).

treatment. These data do not necessarily prove that such antiaromatase agents work through tumour aromatase, which might simply be a marker of hormone dependence, or reflect oestrogen synthesis in other peripheral tissues where the primary action of aminoglutethimide resides. However, it would seem logical to consider tumours which possess both oestrogen synthetic capacity and oestrogen receptors as candidates for therapy with aromatase inhibitors.

It has been suggested that local oestrogen biosynthesis in breast cancers may represent a mechanism whereby they can escape from normal hormonal control (Abul-Hajj 1975). There is little evidence that with increasing autonomy breast cancers are more likely to express aromatase activity, but escape from hormonal control might occur by selection if cells requiring smaller amounts of oestrogen survive and grow on the oestrogen produced as a result of their own aromatizing potential. This residual tumour might be expected to respond to treatment with aromatase inhibitors and, indeed, tumours responding to a previous endocrine treatment are likely to show a further response to aminoglutethimide therapy.

Oestrogen Metabolism

In comparison with oestradiol, oestrone sulphate has low biological potency, but it is the major circulating oestrogen in postmenopausal women (Samojlik *et al.* 1982; Vignon *et al.* 1980). Oestrone sulphate also represents a major precursor of tumour oestradiol in that the enzymes required for the transformation of oestrone sulphate

into oestradiol are present in breast cancers (Wilking *et al.* 1980). This pathway therefore provides another potential important source of active oestrogen.

A recent study has suggested that, at least *in vitro*, the activity of the sulphatase enzyme performing the initial hydrolysis of oestrone sulphate is quantitatively more significant in breast cancers than the aromatase activity associated with *de novo* synthesis of oestrogen (Santner *et al.* 1984). There is, however, a problem in assessing the importance of oestrogen sulphatase in breast cancer in that certain tumours also possess oestrogen sulphotransferase activity. These tumours, therefore, have the potential to perform the reverse reactions, synthesizing oestrone sulphate from free oestradiol. Thus, it is necessary to determine in which direction this metabolic pathway normally proceeds in breast cancers.

A study in which breast cancers were simultaneously incubated with both oestradiol and oestradiol sulphate showed that hydrolysis of oestrogen conjugates is favoured and that the activity of oestrogen sulphotransferase is insufficient to reduce active oestrogen levels (Tseng *et al.* 1983). This suggests that tumour-sulphatase activity represents a major factor controlling concentrations of intracellular oestrogen, despite the lack of correlation between levels of the enzyme and tumour oestrogen and progesterone receptors (Table 12.2). However, it is worth emphasizing that oestrogen sulphotransferase is sensitive to hormones such as progesterone (Buirchell & Hähnel 1975) and that *in vitro* incubation in the absence of hormones or with inappropriate concentrations of cofactors might produce misleading estimates of the enzyme's activity *in vivo*. It is also difficult to ignore the association of oestrogen sulphotransferase with steroid receptors (Table 12.2) and response to endocrine therapy in patients with advanced breast cancer (Dao & Libby 1972).

CONCLUSION

Steroid metabolism within breast tissue is of especial importance because of the sensitivity of this tissue to many of the resultant metabolites. Local synthesis is likely to be most significant when the supply of preformed active hormone reaching the breast declines after the menopause, although cystic tissue with its associated high levels of conjugated steroid precursors may represent an exception.

The relatively high metabolic activity of breast cancers and the abundance of fat within the breast mark these tissues as primary sites of biosynthesis. However, because pathways of steroid inactivation also exist, the balance between synthesis and degradation, particularly of oestrogens and oestrogenic hormones, might determine whether cellular growth is stimulated or inhibited. It is therefore important to identify the, as yet unknown, factors influencing steroid metabolism within the breast so that the potential for local control can be exploited.

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Steroid metabolism in breast cancer

W R Miller

Steroid hormones which may influence the growth and development of both normal and diseased breast are classically synthesized from cholesterol along the pathways shown in Figure 1. Initially, such biosynthesis was thought to be exclusive to the gonads, adrenal cortex and foetal-placental unit, but it is now realized that other organs, including the breast and its tumours, perform many of the transformations. Such potential may have an important influence on the steroid environment within the breast.

The evidence for the existence of such steroid metabolism will be reviewed for breast cancer, benign breast disease, normal breast tissue and breast fat.

Steroidogenesis in breast cancer

Whilst the evidence that breast cancers can synthesize active hormones from cholesterol is unconvincing,^{1,2} there is no doubt that they can use partially-formed precursors which have been produced elsewhere and released into the circulatory system. These "pro-hormones" include androgens, such as dehydroepiandrosterone (DHA) sulphate, which are produced by the adrenal cortex³ and may accumulate in the breast in high concentrations.⁴ Results of studies involving the *in vitro* incubation of tumour^{5,6} or perfusion of the breast *in situ*⁷ suggest that most, if not all, breast cancers metabolise DHA sulphate to other androgens such as DHA itself, Δ 4-androstenedione and testosterone. These metabolites are, in turn, the more immediate precursors of active hormones. For example, DHA may be converted to Δ 5-androstenediol,⁸ a product with oestrogenic activity, and testosterone may be transformed into potent androgens such as 5 α -dihydrotestosterone

and 5 α -androstenediols.^{9,10} All breast cancers have the capacity for 5 α -reduction but the degree of activity varies greatly between individual tumours.¹¹ This appears to be related to the degree of apocrine differentiation within the cancer, those with marked apocrine differentiation showing enhanced production of 5 α -reduced metabolites.¹²

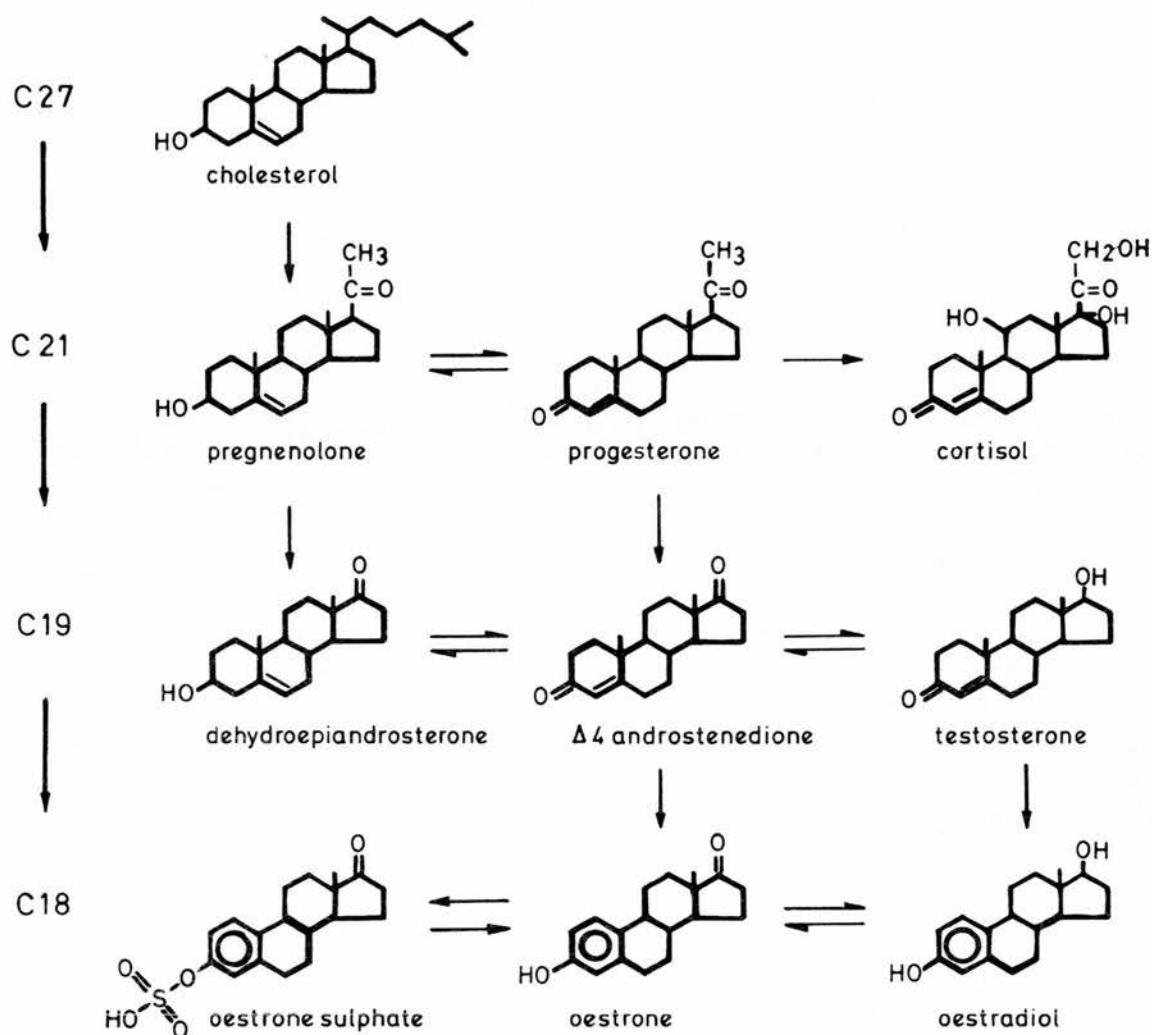
Testosterone and Δ 4-androstenedione are the immediate precursors of oestrogen (Fig. 1) and the results of several groups of investigators have demonstrated the process of aromatization — the conversion of androgens into oestrogens by human breast cancer cells.¹³⁻¹⁷ The proportion of cancers which has this potential varies from approximately 50% to 100% in different reported series, and by using a method of rigorous characterization we have obtained evidence of oestrogen production in over one half of breast cancers.¹⁸ The levels of oestrogen synthesized are small in comparison with those in classical endocrine organs such as the ovary and placenta, but are comparable with, or higher than, levels in other peripheral organs with oestrogen biosynthetic activity.^{15,19}

Because other cells, such as adipocytes, may also produce oestrogen (see below), it is important to demonstrate that oestrogen biosynthesis in specimens of tumour tissue is truly associated with cancer cells. In this respect, studies of cell lines of breast cancers are of value and it is clear that, under optimal culture conditions, breast tumour cells will synthesize oestrogen from androgen precursors.²⁰ Furthermore, different clones of malignant cells from the same tumour may display a differing potential for oestrogen production, thereby demonstrating heterogeneity of aromatase activity in cells from a single tumour.²¹

Breast cancers also have the capacity to

Figure 1

The major pathways of steroid hormone biosynthesis.



interconvert oestrogens (Fig. 1). Oestrone and its sulphate represent the major (although relatively inactive) circulating oestrogens in postmenopausal women,²² but both may be metabolised by breast cancers into the more potent hormone, oestradiol.²³⁻²⁵ Equally, however, many breast cancers can inactivate oestradiol by performing the reverse conversions to oestrone^{23, 26, 27} or to oestrogen conjugates.^{28, 29} The balance of these oestrogen interconversions might be critical in determining the net oestrogenicity potential within the tumour.

Steroidogenesis in benign breast disease

Benign breast disease encompasses a variety of lesions. Whilst there is no evidence that any type of benign breast tissue can transform cholesterol into

steroid hormones, interconversions of androgen and oestrogen, such as those described for breast cancer, occur also in several types of benign lesions.^{9, 10, 17, 23, 26, 30} In general, the transformations occur to a lesser extent than in malignant tissue. The exception is 5 α -reduction in cystic disease³⁰ in which high levels of metabolites may be a reflection of associated apocrine activity.

High levels of steroid hormones may be found in fluids aspirated from breast cysts^{31, 32} where, for example, the median value of DHA sulphate is about 50-fold higher than that in plasma.³³ As high levels of oestrogen conjugates also are present in cyst fluid but are not concentrated from plasma, it has been inferred that these may be synthesized locally from androgen precursors.³⁴ However, no direct evidence for aromatization by breast cysts

has been documented.

Steroidogenesis in normal breast tissue

Specimens of normal breast tissue taken from mastectomy specimens in an area far removed from any malignancy, show evidence of steroidogenic potential.^{6, 10, 17, 26, 30} The metabolism of progestogens, androgens and oestrogens has been reported. The levels of conversion in normal breast tissue are generally lower than those in benign or malignant breast tissues, and this may be due in part to the low cellularity of "normal" resting breast tissue.

Although it is possible that the normal breast may synthesize oestrogens, most workers have not detected a steroid-aromatizing potential in normal breast tissue.^{11, 15, 35} The only group of investigators to report oestrogen biosynthesis described activities in normal tissue which were much lower than those found in parallel incubations of malignant tissue.¹⁹ The general inability to detect oestrogen biosynthesis in normal breast tissue may be due to its low cellularity. Studies of cell lines in culture should help to overcome this problem and, whilst an earlier study failed to show aromatization,²⁰ recently, biosynthesis has been detected in epithelial cells derived from human milk.³⁶

Steroidogenesis in breast fat

In postmenopausal women, aromatization in peripherally-distributed fat probably provides one of the major sources of circulating oestrogen,³⁷ and there are positive correlations between body weight, degree of obesity and levels of plasma oestrogens.^{38, 39} Samples of fat from various parts of the body, including the breast, have been shown to synthesize oestrogen *in vitro*.^{40, 42} In view of the relative increase in the proportion of fat to parenchyma in the breast after the menopause,⁴³ local production of oestrogen by breast fat may be particularly important in the case of postmenopausal women.

Control of steroid metabolism in the breast

As in the case of classical endocrine organs, steroidogenesis in the breast is probably primarily controlled by polypeptide hormones, but the particular hormones involved are yet to be identified. Thus, whilst prolactin is trophic for the breast,⁴⁴⁻⁴⁶ drug-induced hyperprolactinaemia in patients with breast cancer is not associated with differences in steroid metabolism either by normal or malignant breast tissue.⁴⁷

The metabolism of steroid hormones is also modulated by the steroids themselves.⁴⁸ For example, progesterone appears to increase 17 β -hydroxysteroid dehydrogenase activity in breast fibroadenomas⁴⁹ and would account for the maximum enzyme activity found in normal breast tissue during the early secretory phase of the menstrual cycle.⁵⁰ It has also been suggested that 5 α -reduced androgens, which are inhibitors of oestrogen biosynthesis, might provide an intracellular control of aromatase activity in breast cancers.⁵¹

A marked induction of aromatase activity occurs in cultures of cells from either breast cancer or adipose tissue in the presence of cortisol and dexamethasone.^{52, 53} Whether such hormonal effects apply only to the given culture conditions or are reproduced also *in vivo*, is not known.

Relationship to steroid receptors

There has been considerable interest shown in the possible association between oestrogen biosynthesis and oestrogen receptors. It is clear that oestrogen production may be demonstrated in both receptor-positive and receptor-negative tumours.^{14, 16, 18} There is, however, no consensus of opinion about whether oestrogen receptor-positive tumours are more or less likely to produce oestrogen than their receptor-negative counterparts.

Several factors may account for the disparity between results; these include different cut-off values for defining oestrogen receptor-positive tumours, different substrates and methods for assessing tumour aromatase, and the variation in detection limits associated with different characterization techniques for oestrogen. Our own studies¹⁸ showed that within the group of oestrogen receptor-positive tumours, the level of receptor was significantly higher in cancers producing oestrogen than in those without biosynthetic activity. However, the level of oestrogen synthesis does not generally differ between oestrogen receptor-positive and receptor-negative tumours.^{16, 18, 54}

Other aspects of oestrogen metabolism in tumours have been shown to be significantly related to the presence of oestrogen receptors. Thus, there are higher levels of the oestradiol 17 β -dehydrogenase enzyme (which converts oestradiol into oestrone) in oestrogen receptor-poor tumours, compared with those which are receptor-rich.²⁷ Several groups have shown that

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there is also a positive correlation between the ability of tumour cells to sulphate oestrogen and the presence in the tumour cells of oestrogen receptors.^{29, 55, 56}

Conflicting data have emerged over the relationship between oestrogen receptors and the production of 5α -reduced androgens. Abul-Hajj⁵⁷ observed, in a small number of tumours, a positive correlation between these parameters, whereas our investigations on a larger series of cancers derived from postmenopausal patients alone, showed higher 5α -reductase activity in oestrogen receptor-negative tumours.⁵⁸ No significant relationship has been reported between levels of tumour androgen receptors and the production of the natural binding steroid, 5α -dihydrotestosterone.

Biological significance of steroidogenesis in breast cancer

If steroid metabolism within the breast is of biological importance, its role must be local within the breast itself. Effects at sites distant from the breast are improbable because of the relatively low levels of steroid interconversions within breast tissues and because, unlike the case of lung tumours,⁵⁹ widespread responses attributable to inappropriate hormone production have not been reported in patients with breast cancer.

Local hormone biosynthesis is unlikely to be significant in premenopausal patients with breast cancer, in whom the ovary releases sufficient steroid hormones into the circulation to account for the maintenance of hormone-dependent neoplastic growth. It may be of greater importance in the case of premenopausal cystic disease, in which high levels of steroid conjugates are present within the cyst fluid.^{31, 32} Even a small conversion of these inactive precursors into more active products might be associated with marked changes in the hormonal milieu within the breast.

In postmenopausal women, steroid metabolism by the breast may have particular relevance to patients with hormone-dependent breast cancer. After the menopause, the ovary ceases to produce oestrogen and progesterone, although it still secretes some androgen.⁶⁰ The adrenal cortex also synthesizes little oestrogen⁶¹ and, whilst it produces large amounts of androgens such as androstenedione, dehydroepiandrosterone and its sulphate,^{61, 62} these steroids appear to be largely inactive within the breast. Nevertheless, such androgens may be metabolised at peripheral sites, including the breast, into hormones which do have

biological effects on breast cells. It is pertinent, therefore, to consider further the biological significance to breast cancer of some specific aspects of the local metabolism of androgens and their metabolites (Fig. 2).

Synthesis of $\Delta 5$ -androstenediol

The biological importance of $\Delta 5$ -androstenediol resides in its oestrogenic activity. The steroid binds to oestrogen receptors,⁶³ and is capable of eliciting oestrogen-mediated responses, eg, the induction of progesterone receptors.⁶⁴ The concentrations of $\Delta 5$ -androstenediol required for these effects are about 2nM,⁶³ which is about 40-times higher than the level of oestradiol necessary for the same response. Thus, where oestradiol levels are low in comparison with androgens (as, for example, in postmenopausal women) $\Delta 5$ -androstenediol may act as an oestrogen, or at least supplement the stimulus of natural oestrogens.

Results of measurements of the relative levels of $\Delta 5$ -androstenediol in tumours^{63, 65} support the concept that the steroid has an oestrogenic potential in breast cancers in postmenopausal women, but it is not clear whether the tumour concentrations of $\Delta 5$ -androstenediol result from local synthesis. Breast cancers certainly have the capacity to convert DHA and its sulphate to $\Delta 5$ -androstenediol^{6, 63} and there are large amounts of these precursors in the breast.⁴ Whilst both free and conjugated DHA have very little oestrogenic activity,⁶³ their conversion to $\Delta 5$ -androstenediol would increase the oestrogenic environment of the tumour.

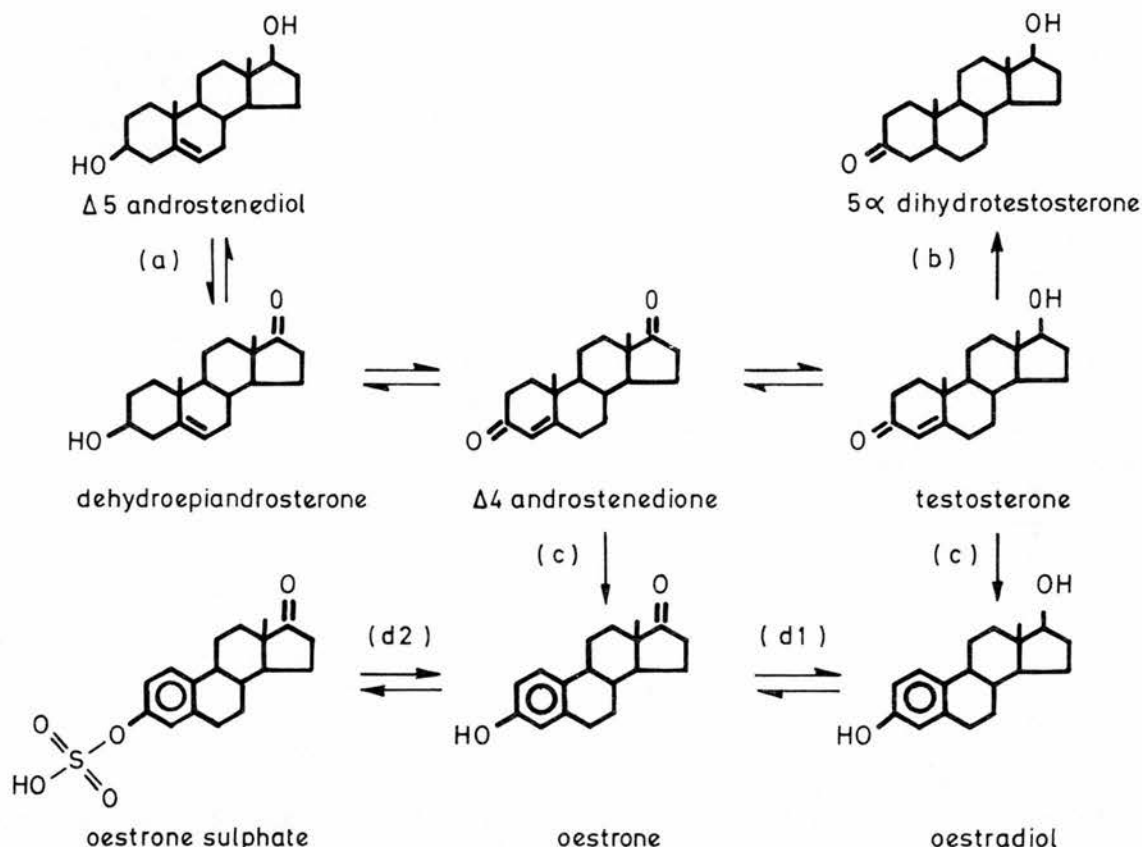
5α -reduction

5α -reduced androgens are amongst the most potent androgenic steroids, and 5α -dihydrotestosterone forms the natural ligand for androgen receptors. Tissues which are sensitive to androgens invariably show high 5α -reductase activity⁶⁶ and breast tissues may show a high potential for 5α -reduction. About 30-40% of breast cancers possess cytoplasmic androgen receptors^{67, 68} but no correlation has been shown between 5α -reduction and the presence of androgen receptors in tumours, and anti-androgen therapy has produced few advances in the treatment of breast cancer.

If, therefore, 5α -reductase activity in the tumour is of biological significance, it is likely to be independent of androgen receptors. For example, since 5α -reduced steroids are powerful inhibitors

Figure 2

Metabolic transformations producing major changes in biological activity of steroids (a) formation of the oestrogenic androgen, $\Delta 5$ -androstenediol (b) synthesis of the potent androgen, 5α -dihydrotestosterone (c) aromatization of androgens to oestrogens (d) interconversion of oestrogens either by 17β -dehydrogenase (1) or sulphokinase/sulphatase (2).



of aromatase activity,⁴⁸ 5α -reductase activity in the tumour might control oestrogen biosynthesis *in situ*. In support of this hypothesis, an inverse correlation has been reported between 5α -reduction and aromatase activity in breast cancer cells.⁵¹ Some synthetic androgens, which have been used successfully in the treatment of advanced breast cancer, have also subsequently proved to be aromatase inhibitors.⁶⁹

Aromatase activity

The potential importance of tumour aromatase is that oestrogen-dependent tumours may satisfy their hormone requirements by their own capacity to synthesize oestradiol. However, whilst it is clear that over 50% of breast cancers has the potential for aromatization, there are doubts as to the relevance of the small levels of activity detected in the majority of tissue samples incubated *in*

vitro.^{54, 70} Theoretical calculations based on *in vitro* conversion rates, estimates of available androgen precursor and the concentration of oestrogen required to interact with oestrogen receptors, suggest that tumour aromatase produces an effective level of oestrogen in only about 10% of all breast cancers. This could be of significance if these tumours were among the one-third of cancers which respond to oestrogen deprivation therapy.

There are of course, problems in interpreting results quantitatively from *in vitro* studies designed to detect *potential* for aromatization rather than to reflect conditions *in situ*. For example, little account has been taken of the high concentrations of androgen conjugates within the breast which could supplement the oestrogen precursor pool. Equally, concentrations of oestrogen substantially less than those required to saturate oestrogen receptors can

maintain the growth of hormone-dependent cells.⁷¹ Until comparative studies of tumour aromatase have been performed in cancers with high and low endogenous concentrations of oestrogen, the relative contribution of local biosynthesis to the pool of active hormone cannot accurately be assessed.

The presence of aromatase activity in both oestrogen receptor-positive and receptor-negative tumours,^{14, 16, 18} and the lack of any relationship between aromatase and progesterone-receptor status⁵⁴ does not necessarily preclude the physiological significance of tumour oestrogen biosynthesis. Aromatase activity may have a biological role independent of receptors, or it may be of significance only in steroid receptor-positive tumours. Abul-Hajj⁷² has advocated the former possibility, suggesting that oestrogen receptor-negative tumours appear endocrine-independent only because they can satisfy their hormone needs by means of their own biosynthetic capacity. This seems unlikely, because tumours responding to therapy using aromatase inhibitors or anti-oestrogens (which ought to be effective irrespective of the source of oestrogen), are invariably oestrogen receptor-positive.^{73, 74} It is much more likely that if oestrogen biosynthesis is of significance, it will be in oestrogen receptor-positive tumours in which a mechanism exists to process the synthesized steroid.

It has been suggested that local oestrogen biosynthesis in breast cancers may provide a mechanism whereby the tumours can escape from normal hormonal control.⁷² Whilst there is little evidence that, with increasing autonomy, breast cancers are more likely to express aromatase activity, escape from hormonal control might occur by a selection process if cells requiring smaller amounts of oestrogen survive, and are stimulated by the oestrogen produced as a result of their own aromatizing potential. This residual tumour might be expected to respond to treatment with aromatase inhibitors and indeed, tumours responding to a previous endocrine treatment are likely to show a further response to aminoglutethimide therapy.⁷³

Oestrogen metabolism

In comparison with oestradiol, oestrone sulphate has a low biological potency, but it is the major oestrogen in the circulatory system of postmenopausal women.^{22, 24} Oestrone sulphate also represents a major precursor of tumour oestradiol, in that the enzymes required for the

transformation of oestrone sulphate into oestradiol are present in breast cancers.²⁵ This pathway, therefore, provides another potentially important source of active oestrogen.

Results of a recent study have suggested that, at least *in vitro*, the activity of the sulphatase enzyme performing the initial hydrolysis of oestrone sulphate is quantitatively more significant in breast cancers than the aromatase activity associated with the *de novo* synthesis of oestrogen.⁷⁵ There is, however, a problem in assessing the importance of oestrogen sulphatase in breast cancer, in that certain tumours also possess oestrogen sulphotransferase activity. These tumours, therefore, have the potential to perform the reverse reaction, synthesizing oestrone sulphate from free oestradiol. Although sulphatase activity appears to predominate in disrupted cell preparations,⁵⁶ it is still not clear in which direction the metabolic pathway normally proceeds in breast cancers *in situ*.

Conclusion

Steroid metabolism within the breast is of especial importance because of the sensitivity of breast tissue to many of the resultant metabolites. Local synthesis is likely to be most significant when the supply of preformed active hormone reaching the breast declines after the menopause, although cystic tissue, with its associated high levels of conjugated steroid precursors, may represent an exception.

Steroid metabolism in breast cancers and breast fat merits particular interest. Pathways of steroid activation and inactivation exist and the metabolic balance might determine whether cell growth is stimulated or inhibited. Because of this, it is important to identify the factors controlling steroid metabolism within the breast. It may then be possible to harness the potential to modify the intracellular environment of hormones and thereby influence growth.

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SECTION B

STEROID METABOLISM BY

(A) RAT MAMMARY TUMOURS

AND

(B) OTHER HUMAN TISSUES

STEROID METABOLISM BY HUMAN BREAST AND
RAT MAMMARY CARCINOMATA

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ABSTRACT

The metabolism of dehydroepiandrosterone (DHA) and testosterone by both human breast carcinomata and dimethyl-benzanthracene (DMBA)-induced rat mammary carcinomata has been investigated.

The rat and human carcinomata converted DHA to testosterone and both DHA and testosterone to 5α -dihydrotestosterone, 5α -androstenediol and 16α -hydroxytestosterone. Tentative evidence is also presented to indicate that some rat adenocarcinomata can convert androgen precursors into estradiol- 17β .

Although quantitative differences between incubations occurred, the spectrum of steroid transformations was similar in both human and rat tumours. The DMBA-induced rat tumour may therefore prove to be a valuable experimental model for human carcinoma tissue with regard to further steroidogenic studies.

INTRODUCTION

The DMBA-induced rat tumour has many characteristics which are similar to those possessed by human breast carcinoma. Many of the tumours are hormone responsive ⁽¹⁾, and some possess an estrogen receptor protein similar to that found in human carcinomata ⁽²⁾, a property correlated with their hormone

responsiveness (3). It has also been shown that the DMBA-induced rat carcinoma is capable of performing steroid conversions, metabolising both DHA and testosterone (4,5). Recently similar paraendocrine behaviour has been demonstrated in human carcinomata (6,7). The present paper describes a direct comparison of human and DMBA-induced rat carcinomata by identical methods to determine their capacity to transform DHA and testosterone into steroids which may influence tumour growth.

MATERIALS AND METHODS

Steroids

(7 α -³H)-DHA and (4-¹⁴C)-testosterone were obtained from the radiochemical centre (Amersham) and were found to be chromatographically homogeneous in the major solvent systems used. Immediately before use, however, both precursors were purified by thin layer chromatography (TLC) in solvent system I (Table 1).

Unlabelled steroids were obtained from Sigma Ltd., Kingston-upon-Thames, Surrey; Steraloids Ltd., Croydon, Surrey; and Ikapharm Ltd. (Israel). The MRC steroid reference collection generously donated samples of 16 α -hydroxytestosterone.

Human Tumour Tissue

Breast carcinoma tissue was obtained at operation from three patients. Patient I was aged 50 years, the breast tumour weighed about 1 g. and was histologically intraduct cancer with a small focus of invasion. Patient II was aged 40 years. The tumour was large (approximately 20 g.) and histologically was a non-differentiated carcinoma of large cell type. Patient III was aged 50 years. The tumour weighed about 4 g. and histologically was a carcinoma of unknown origin (possibly apocrine).

Rat Tumour Tissue

Tumours were induced in five 50 day-old female Sprague Dawley rats with a single dose of dimethylbenzanthracene (30 mg. in 2 ml. cottonseed oil) given by gastric intubation. Tumours were collected after killing the rats by cervical dislocation. Histology of the tumours revealed a squamous carcinoma in Rat II and adenocarcinoma in all other rats (two adenocarcinomata were present in anatomically different regions in Rat I - (a) from the inguinal area, and (b) from the cervical area).

Preparation of tumour tissue and conditions of incubation

The tumours were maintained at 0°C after removal and incubations were carried out within 30 minutes of removal. Any adhering fat and normal mammary tissue was removed. The tumours were then finely sliced. Krebs-Ringer phosphate buffer pH 7.4 (10 ml/g. tumour) was added and sonicated twice for 1 minute with cooling for 1 minute between sonications. A NADPH generating system (200 μ mol. glucose-6-phosphate, 25 mg. NADP and 50 units glucose-6-phosphate dehydrogenase) was added before the addition of the purified radioactive precursors (about 20 μ c (7α - ^3H)-DHA and 2 μ c (4 - ^{14}C)-testosterone in 0.1 ml. propylene glycol:ethanol, 1:1). The tumour slices were then incubated by shaking at 37°C for two hours in an atmosphere of O_2 . The reaction was stopped by adding methanol to 80% (v/v) and the incubations stored at -10°C until processed.

Extraction and Fractionation of steroids

Before extraction, 500 μ g of the non-radioactive carrier steroids to be investigated were added. The mixture was then homogenized, centrifuged and the resulting supernatant decanted off. The residue was rehomogenised with 2 x 20 ml. acetone and the acetone washings were combined with the aqueous methanol supernatant and evaporated to dryness. This residue was dissolved in aqueous methanol and separated into neutral and phenolic fractions as described by Fahmy, Griffiths, Turnbull and Symington ⁽⁸⁾.

Characterization of Metabolites

Chromatographic Separation of Steroids

Thin layer chromatography on silica gel HF 254+366 and alumina PF 254+366 was used to isolate and purify the individual steroids. The fractionation procedure for the neutral steroids is shown in figure 1. The solvent systems used are summarised in Table 1. The phenolic fraction was separated into estrone and estradiol-17 β fractions by TLC in system VIII. The individual estrogens were run as the acetates in TLC system IIIa and then, after saponification, in system IX.

The steroids were detected on the plates by absorption of U.V. light at 254 or 350 nm and eluted with ethanol after deactivation of the gel with a few drops of distilled water.

Microchemical reactions

Acetates were prepared by the method of Zaffaroni and Burton (9).

Saponification of the steroid acetates and oxidation of free steroids were performed as described by Griffiths *et al* (10).

Reduction of steroids was performed by adding 10 mg. solid sodium borohydride to the steroid dissolved in 1 ml. methanol and leaving the mixture for 15 min. at room temperature.

In all cases steroids were recovered by extraction with 2 x 5 ml. ethyl acetate after the addition of 3 ml. distilled water.

Measurement of radioactivity

Radioactivity was measured with a Nuclear-Chicago liquid scintillation counter Mark I Model 6860. Counting conditions were such that ^{14}C and ^3H could be determined simultaneously with efficiencies in channel I and II, respectively, of 12% and 52% for ^{14}C , and 45% and 0% for ^3H . The absolute quantities of ^3H were calculated from readings in channel I after correction for ^{14}C counts (11) and those for ^{14}C from direct readings on channel II, no ^3H counts being present in this channel under the conditions specified. No quenching was detected.

TABLE ITLC SOLVENT SYSTEMS AND CONDITIONS USED TO
PURIFY AND IDENTIFY STEROIDS

SYSTEM NO.	SOLVENTS (PROPORTIONS BY VOLUME)	CONDITIONS
I	Chloroform: acetone (98:2)	Continuous elution tank 3½ hours
II	Chloroform: acetone (185:15)	
IIIa	Cyclohexane: ethyl acetate (70:30)	
b	Cyclohexane: ethyl acetate (70:30)	Continuous elution tank 3½ hours
IV	Chloroform: ether: acetone: cyclohexane (10:50:1:140)	
V	Chloroform: ether: acetone: hexane (10:50:6:140)	Alumina plate. Continuous elution tank, overnight
VI	Ethylacetate: <u>n</u> -hexane: acetic acid: alcohol (72:13.5:10:4.5)	
VII	Ethylacetate: <u>n</u> -hexane: acetic acid (75:20:5)	
VIII	Cyclohexane: ethyl acetate (55:45)	
IX	Hexane: ethyl acetate (50:50)	
X	Dichlormethane: cyclohexane: acetone (50:30:10)	
XI	Cyclohexane: ethyl acetate (50:50)	

Unless stated otherwise, silica gel HF 254+366 plates were used in a Shandon Chromatank. The solvent was run to the top of the plate (running time approximately 1 hr.).

Table 2
EVIDENCE FOR THE IDENTIFICATION OF STEROIDS ISOLATED FROM HUMAN TUMOUR INCUBATIONS

Steroid isolated	Derivative	Compared with authentic steroid in solvent system	Specific Radioactivity dpm / n mol.					
			Human I		Human II		Human III	
			H ³	C ¹⁴	H ³	C ¹⁴	H ³	C ¹⁴
DHA	DHA	I II IIIb	20,500	3.54	*	*	13,400	1.36
	DHA acetate	IIIa III	19,800	4.16	*	*	13,300	1.45
	5 - androstenediol	II	20,600	3.85	*	*	13,400	1.51
Testosterone	Testosterone	I II V	42.4	2,600	59.0	2,420	109	2,650
	Testosterone acetate	IIIa	55.0	2,400	54.4	2,210	109	2,670
	4 - androstenedione	II	51.2	2,680	55.1	2,370	100	2,540
5 α -Dihydrotestosterone	5 α DHT	I II IIIb	0	10.8	69.9	22.4	23.9	69.6
	5 α DHT acetate	IIIa IV	0	12.1	69.8	22.0	23.9	73.2
	5 α - androstenediol	II	0	11.3	63.4	21.0	21.9	71.0
5 α -Androstenediol	5 α -androstenediol	I II IX	—	—	—	—	51.5	13.7
	5 α - androstenedione	II IX	—	—	—	—	52.2	12.9
16 α OH - Testosterone	16 α OH - Testosterone	VI VII	2.60	0.52	3.0	0.34	3.12	0.32
	16 α OH - Testosterone diacetate	II	2.60	0.54	3.1	0.33	3.02	0.40

* Fraction lost — Not investigated

Estradiol - 17 β and Estrone examined in each incubation but insufficient counts found to characterize

Table 3
EVIDENCE FOR THE IDENTIFICATION OF STEROIDS ISOLATED FROM RAT TUMOUR INCUBATIONS

Steroid isolated	Derivative	compared with authentic steroid in solvent system	Specific Radioactivity dpm / n mol.											
			Rat I(a)		Rat I(b)		Rat II		Rat III		Rat IV		Rat V	
			H ³	C ¹⁴	H ³	C ¹⁴	H ³	C ¹⁴	H ³	C ¹⁴	H ³	C ¹⁴	H ³	C ¹⁴
DHA	DHA	I II IIIb	15,600	184	16,600	160	20,600	694	24,600	230	25,100	172	20,600	459
	DHA acetate	IIIa IV	16,000	172	17,500	162	21,100	621	24,100	208	23,700	191	21,900	475
	5 - androstenediol	II	14,900	200	16,700	197	22,200	698	25,900	246	24,000	159	22,200	428
Testosterone	Testosterone	I II V	117	1,810	130	1,750	120	2,030	688	2,270	365	1,100	180	1,730
	Testosterone acetate	IIIa	115	1,720	141	1,860	120	1,980	749	2,210	368	1,100	184	1,700
	4 - androstenedione	II	119	1,710	144	1,840	132	1,950	773	2,320	438	1,050	195	1,800
5 α -Dihydrotestosterone	5 α -DHT	I II IIIb	288	125	130	159	45.0	162	0	162	0	402	0	210
	5 α -DHT acetate	IIIa IV	293	126	140	158	35.4	156	0	166	0	385	0	195
	5 α - androstenediol	II	265	119	139	154	38.4	14.9	0	170	0	389	0	198
5 α -Androstenediol	5 α -androstenediol	I II IX	-	-	-	-	-	-	291	209	285	370	0	64.0
	5 α -androstenedione	II IX	-	-	-	-	-	-	270	226	265	372	0	61.0
16 α -OH Testosterone	16 α -OH Testosterone	VI VII	0	0	4.53	1.04	8.65	1.81	0	2.10	0	0	0	4.00
	16 α -OH Testosterone diacetate	II	0	0	4.68	1.12	8.43	1.85	0	2.00	0	0	0	4.43
Estradiol - 17 β	Estradiol - 17 β	VIII IX	3.78	0.27	3.68	0.21	0	0	0	0	0	0	0	0
	Estradiol - 17 β diacetate	III a	3.63	0.23	3.61	0.21	0	0	0	0	0	0	0	0

- Not investigated
Estrone was examined in each incubation but insufficient counts found to characterize

Table 4

METABOLISM OF $7\alpha^3\text{H}$ DHA BY HUMAN AND RAT TUMOURS

Steroid	Human I	Human II	Human III	Rat I (a)	Rat I (b)	Rat II	Rat III	Rat IV	Rat V
DHA	75.50	*	65.40	76.90	83.40	82.20	82.16	81.06	83.40
Testosterone	0.18	0.16	0.53	0.58	0.68	0.50	0.24	0.13	0.72
5α -Dihydrotestosterone	0	0.19	0.11	1.39	0.65	0.15	0	0	0
5α -Androstane-17 β -diol	—	—	0.25	—	—	—	0.92	0.90	0
16α -OH Testosterone	0.01	0.01	0.01	0	0.02	0.03	0	0	0
Estradiol - 17β	0	0	0	0.02	0.02	0	0	0	0
Estrone	0	0	0	0	0	0	0	0	0

* Fraction lost

— Not investigated

Measurement of unlabelled steroids

Testosterone, 16 α -hydroxytestosterone and derivatives were determined by U.V. absorption at 240 nm.

All other steroids were measured by GLC analysis on a 5 ft. column of 3% OV1 on Gas Chrom Q (100 - 120 mesh) (Applied Science Laboratories Inc.) at 223°C. Testosterone (for free steroids) and testosterone acetate (for acetylated steroids) were used as internal standards.

RESULTS

Identification of steroids

Evidence for the identification of the steroids isolated from the incubations is summarised in Table 2 (Human Tumours) and Table 3 (Rat Tumours).

Characterization is based on (a) derivative formation; (b) comparison of the chromatographic mobilities of the free and derivative steroids with those of authentic steroids; and (c) consistency between the specific radioactivity (S.A.) of the purified parent steroid and its prepared derivatives.

Metabolism of DHA

The distribution of radioactivity among the metabolites is shown in Table 4 and is expressed as percentage of the initial radioactivity added as DHA. The percentage conversions were calculated from the average S.A. of each steroid and its derivatives.

In each incubation most of the ^3H counts remained associated with DHA. Small but significant ^3H labelling was associated with testosterone, 5α -dihydrotestosterone and 5α -androstanediol in both rat and human tumour incubations. The counts found in these metabolites were always in excess of 20 x that of background for testosterone and 5α -androstanediol fractions, and 10 x that of background for 5α -DHT fractions. In each case such ^3H labelling gave consistent specific radioactivities in the parent steroid and derivatives. Similarly the $^3\text{H}/^{14}\text{C}$ ratios were constant in parent steroids and derivatives (Tables 2 and 3).

Low ^3H counts (more than 4 x background) were also found in 16α -hydroxytestosterone fractions from each human tumour incubation and two of the rat tumour incubations. Because of the low radioactivity associated with these fractions, only a single derivative (the diacetate) was prepared for characterization. Nevertheless, the specific ^3H activity and $^3\text{H}/^{14}\text{C}$ ratio were unchanged from parent steroid to derivative.

Similar low levels of ^3H counts were associated with the estradiol- 17β fraction from the incubations of the two tumours from Rat I. The specific ^3H activities and $^3\text{H}/^{14}\text{C}$ ratios were constant for the diacetate derivative and parent estradiol- 17β in both fractions. Counts in the corresponding estradiol- 17β fractions from incubations of human and the other rat carcinomata

CHROMATOGRAPHIC SEPARATION AND PURIFICATION OF NEUTRAL STEROID EXTRACT

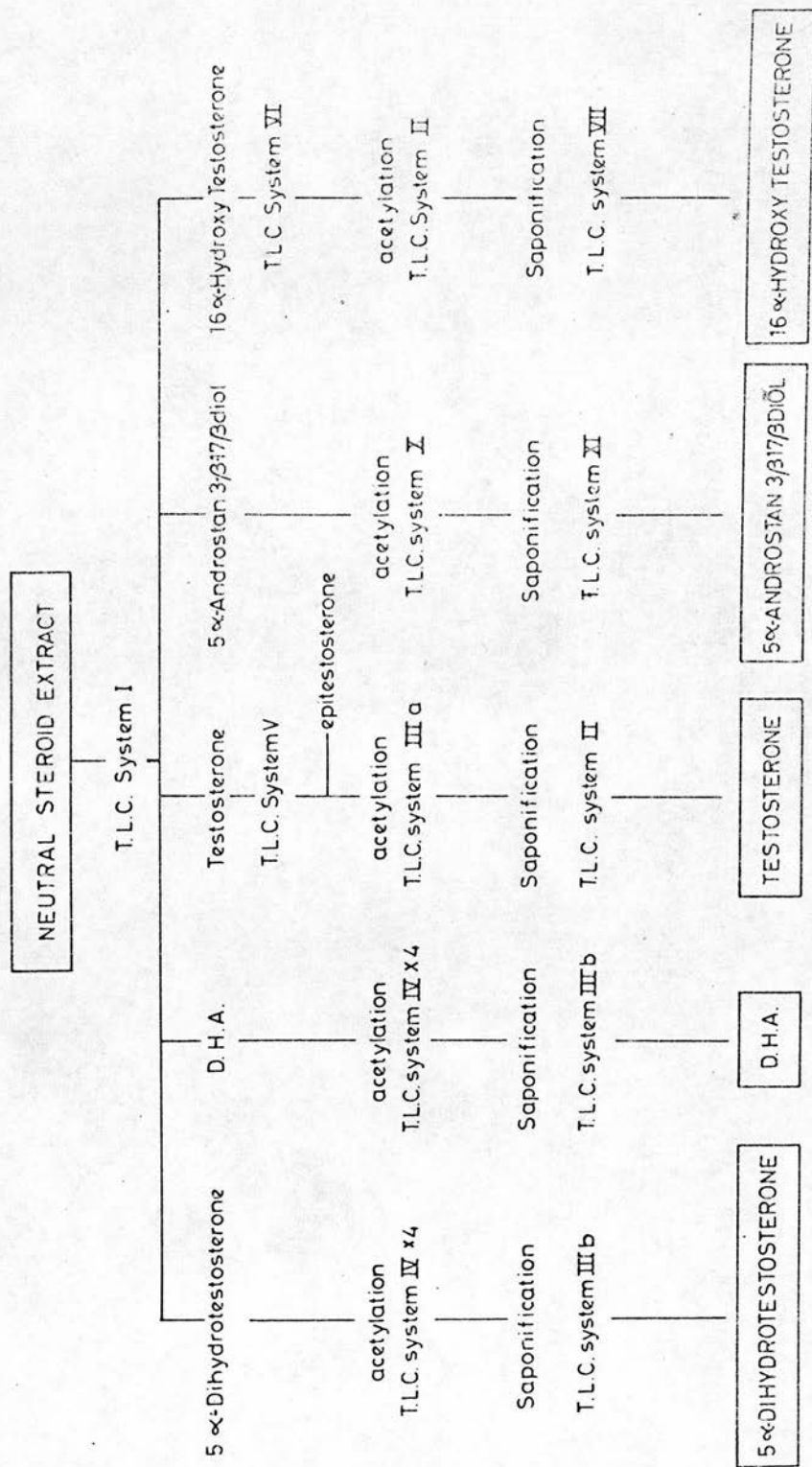


Table 5
METABOLISM OF 4 - ¹⁴C TESTOSTERONE BY HUMAN AND RAT TUMOURS

Steroid	Human I	Human II	Human III	Rat I (a)	Rat I (b)	Rat II	Rat III	Rat IV	Rat V
Testosterone	89.80	87.10	95.45	68.10	71.10	79.60	80.30	38.41	81.60
5 α - Dihydrotestosterone	0.40	0.83	2.62	5.24	6.10	0.62	0.58	13.79	9.30
5 α - Androstane-3 β -diol	—	—	0.49	—	—	—	0.76	1.30	2.90
16 α -OH Testosterone	0.02	0.01	0.01	0	0.04	0.06	0	0.06	0.19
DHA	0.13	*	0.05	0.07	0.06	0.27	0.08	0.57	0.21
Estradiol - 17 β	0	0	0	0.01	0.01	0	0	0	0
Estrone	0	0	0	0	0	0	0	0	0

* Fraction lost
— Not investigated

were insufficient to permit characterization. There was no evidence in any incubation for the formation of estrone.

Metabolism of testosterone

The distribution of radioactivity among the metabolites isolated is shown in Table 5, the percentage conversions being calculated in the same manner as those for the metabolites of DHA.

A large proportion of the ^{14}C counts remained associated with the testosterone precursor in each incubation. There was, however, greater metabolism of the precursor in the incubations of rat tissue. In each incubation there was significant ^{14}C labelling of 5α -DHT fractions, indicating the presence of 5α -reductase activity in the tumours. The level of transformation to 5α -DHT was, however, variable, particularly in the rat tissues. Further evidence for the 5α reduction of testosterone was obtained from 5α -androstenediol fractions. In all incubations where these fractions were examined, significant labelling with ^{14}C was detected.

Small but significant ^{14}C counts (at least 5 x that of background) were associated with 16α -hydroxytestosterone fractions from each human tumour incubation and four of the six rat tumour incubations. The specific ^{14}C activity of these fractions was alike whether the 16α -hydroxytestosterone was assayed as the free or acetylated steroid.

Certain of the DHA fractions (Human I, Rat II, IV and V) were clearly labelled with ^{14}C - evidence for the conversions of testosterone to DHA. Similarly ^{14}C counts were detected in DHA fractions from the other incubations, but at a lower level, which may represent small incorporation from testosterone.

Low levels of ^{14}C counts were associated with estradiol- 17β fractions in the two adenocarcinomata from Rat I. Although the counts were low (4 x background) the ^{14}C specific activity and $^3\text{H}/^{14}\text{C}$ ratio were similar in each purified estradiol- 17β fraction and its diacetate derivative. Radioactivity in the estradiol- 17β fractions from all other incubations was too small to allow characterization. No evidence was found for the formation of estrone from testosterone in any incubation.

Discussion

Several groups of workers (6,7,12) have shown that human breast cancer may act as a paraendocrine organ by metabolising steroid hormones. The present study confirms the work of these authors. It was possible to demonstrate that human breast cancer tissue can convert DHA to testosterone and both DHA and testosterone to 5α -dihydrotestosterone and 5α -androstanediol in significant amounts. 16α -hydroxylase activity was also demonstrable as evidenced by the production of small amounts of

labelled 16α -hydroxytestosterone in the incubations. The magnitudes of these transformations were very similar to those previously reported.

In none of the three human carcinomata examined in the present study was it possible to detect the production of either estradiol- 17β or estrone from the C_{19} steroid precursors. Jones *et al* ⁽⁷⁾, however, have reported a production of estrone from 4-androstenedione (0.005%) and DHA (0.0005%) by human carcinoma. These levels of conversion would be difficult to detect using the methods employed in this study.

The rat mammary tumours induced by intragastric instillation of DMBA were also able to convert DHA to testosterone and both precursors to 5α -DHT, 5α -androstanediol and 16α -hydroxytestosterone. The magnitude of these conversions were close to those reported in similar rat tumours by King, Gordon and Helfenstein ⁽⁴⁾. In the present study, however, there was also some evidence of aromatization of C_{19} steroid precursors in two adenocarcinomata obtained from a single rat. This activity has not been previously reported in the DMBA-induced rat adenocarcinoma.

It is interesting to note that the steroid transformations performed by the two adenocarcinomata obtained from different sites in the same rat were remarkably similar. It may

therefore be possible to pool histologically similar tumours from the same animal in future studies.

The steroid interconversions described are not unique to mammary carcinomata and have been found in normal human breast (13) and mammary tissue from pregnant rats (4,5). The interconversions, however, assume an additional importance in mammary carcinomata as the resulting metabolites may influence the growth of the tumour (1). It is therefore essential to have an experimental animal model for the human carcinoma in order to determine which factors are able to affect steroid metabolism in mammary tumours.

The results from the present study indicate that the DMBA-induced rat carcinoma and human breast cancer are alike in their capacity to metabolize steroid hormones. Although there are quantitative differences between incubations, the spectrum of activity in both rat and human tumours is identical. This is further justification for the use of the DMBA-induced rat tumour as an experimental model for the human breast carcinoma.

ACKNOWLEDGEMENTS

This work was performed with a grant from the Cancer Research Campaign from whom W.R. Miller is in receipt of full-time support. The authors thank Mr. D. McDonald, Miss J. Telford, Mrs. A. Boyd and Mr. I. Ansell for their skilled technical assistance and Mr. R.A. Hawkins for his corrective reading of the manuscript.

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The following trivial names have been used in the text:

Dehydroepiandrosterone, D.H.A. = 3β -hydroxy-5-androsten-17-one

D.H.A. acetate = 3β acetoxy-5-androsten-17-one

5 α -Dihydrotestosterone, D.H.T. = 17β -hydroxy-5 α -androstan-3-one

5 α -D.H.T. acetate = 17β -acetoxy-5 α -androstan-3-one

Testosterone = 17β -hydroxy-4-androsten-3-one

Testosterone acetate = 17β -acetoxy-4-androsten-3-one

16α -hydroxytestosterone = 16α , 17β -dihydroxy-4-androsten-3-one

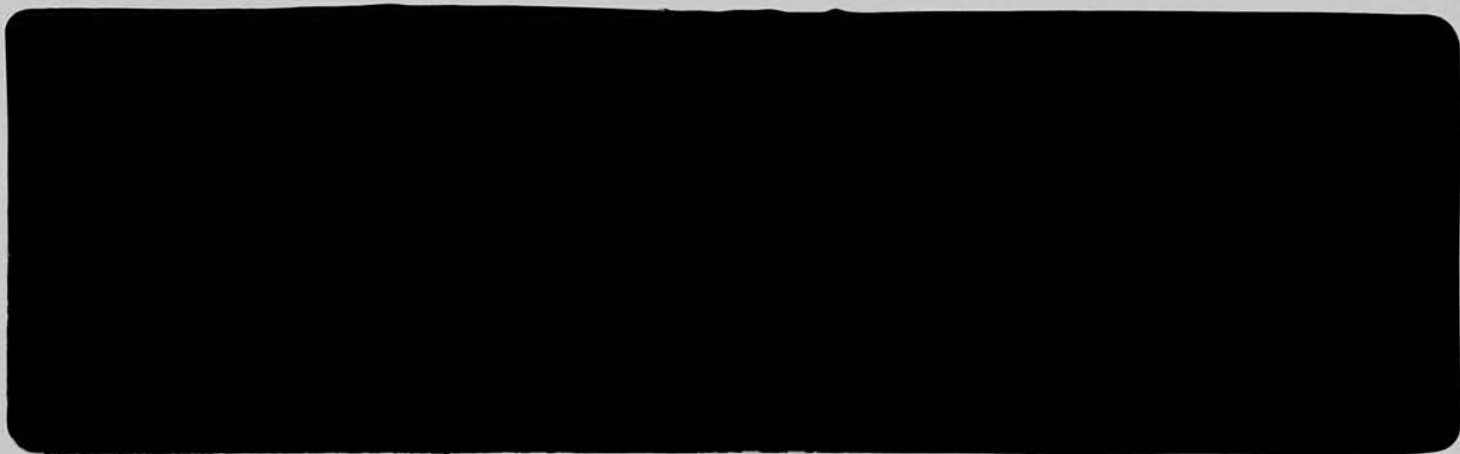
16α -hydroxytestosterone diacetate = 16α , 17β -diacetoxy-4-androsten-3-one

5-androstenediol = 5-androstene- 3β , 17β -diol

4-androstenedione = 4-androstene-3, 17-dione

5 α -androstenediol = 5 α -androstane- 3β , 17β -diol

5 α -androstenedione = 5 α -androstane-3, 17-dione



Hyperprolactinemia and Steroid Metabolism by Rat Mammary Adenocarcinomas¹

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SUMMARY

The metabolism of both testosterone and dehydroepiandrosterone by 10 adenocarcinomas induced and grown in the presence of high prolactin (plasma prolactin > 220 ng/ml) was compared with the metabolism of 10 adenocarcinomas induced and grown in the presence of normal levels (plasma prolactin < 60 ng/ml). The tumors associated with high prolactin significantly metabolized more testosterone to both 5 α -dihydrotestosterone and 5 α -androstenediol. The metabolism of dehydroepiandrosterone was similar in both groups of tumors. It is concluded that prolactin may differentially influence the metabolism of C₁₉ steroids by rat mammary adenocarcinomas.

INTRODUCTION

The growth and development of rat mammary tumors are influenced by prolactin (5, 10, 12). There is an increased occurrence of mammary tumors in rats having high levels of plasma prolactin (1, 14), and elevation of plasma prolactin levels accelerates the growth of mammary tumors (4, 13). Rat mammary tumors also demonstrate paraendocrine activity and metabolize steroid hormones such as testosterone and DHA² (9, 11). The aim of the present study was to determine the effect of raising prolactin levels on tumor steroidogenesis.

MATERIALS AND METHODS

Animals. Two groups of random-bred female Sprague-Dawley rats were studied. One group received s.c. injections of perphenazine (5 mg/kg body weight) from 30 days of age; the other group received daily doses of injection vehicle (0.2% citric acid) from the same age. Both groups of animals received 5 mg 7,12-dimethylbenzanthracene in a single i.v. injection at 50 days of age. The mammary tumors so induced were allowed to grow to a size of 2 × 2 cm. The rats were then sacrificed in diestrus, when the tumors were taken for incubation and blood was drawn for plasma prolactin determination. Subsequent analysis of plasma prolactin allowed the animals to be divided into a group of

animals receiving perphenazine, with values over 220 ng/ml (high prolactin), and a control group receiving injection vehicle, with values below 60 ng/ml (normal prolactin). Ten adenocarcinomas from each group were investigated.

Tumor Incubation. All tumors were processed at 0° until incubation was carried out (within 30 min of tissue removal). One g of each tumor was finely sliced in duplicate, and Krebs-Ringer phosphate buffer, pH 7.4 (10 ml), was added. The tissue was then sonically extracted twice for 1 min and an NADPH-generating system (200 μ moles glucose 6-phosphate, 25 mg NADP, and 50 units glucose-6-phosphate dehydrogenase) and the radioactive precursor (45 μ Ci of either 7 α -[³H]testosterone or 7 α -[³H]DHA) were added. The tumor slices were then incubated by shaking at 37° in an atmosphere of oxygen for 2 hr. The reaction was stopped by adding methanol to 80% (v/v), and the incubations were stored at -10° until processed.

Purification and Characterization of Metabolites. Before extraction, 500 μ g of nonradioactive carrier steroids were added to monitor recovery losses. The metabolites were then extracted, separated into individual steroids, and purified by thin layer chromatography, as described previously (11). Each purified metabolite was characterized by comparing specific radioactivities of parent steroids and authenticated derivatives. The procedures for derivative formation and measurement of cold steroids have been described previously (11). The percentages of steroid metabolism and interconversions were determined by measuring the percentage of incorporation of radioactive label into the appropriate metabolites after correction for recovery losses.

DNA Measurement. The DNA content of the tumors was determined by a modification of the method of Burton (3).

RESULTS

Tumor Induction and Growth. There was a significant difference between the 2 animal groups in their ages at time of sacrifice (when the tumor size was approximately 2 × 2 cm), the mean age \pm S. E. for the high prolactin group being 184 \pm 14 days and that for the normal prolactin group being 226 \pm 12 days. This difference was due to the increased growth rate of tumors from the high prolactin group, there being no difference between the groups in the time period of tumor induction.

Tumor Steroid Metabolism. A comparison of the percentage of metabolism of testosterone by 10 adenocarcinomas induced and grown in the presence of prolactin levels in

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² The abbreviations used are: DHA, dehydroepiandrosterone; 5 α -DHT, 5 α -dihydrotestosterone.

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excess of 220 ng/ml (high prolactin group) with that by 10 adenocarcinomas generated in the presence of values below 60 ng/ml (normal prolactin group) is presented in Chart 1. Despite the large spread of activity in both groups of tumors, the tumors associated with high prolactin tended to metabolize more testosterone, and the difference between the groups was significant ($p < 0.005$). The percentages of conversion of testosterone to 5α -DHT (Chart 2) and 5α -androstanediol (Chart 3) showed a similar pattern, the transformations in the high prolactin group being significantly increased ($p < 0.005$ in each case). When the percentages of production of 5α -DHT and 5α -androstanediol were combined as a percentage of 5α reduction (Chart 4), there was complete separation between the 2 groups of tumors, with all the tumors from the high prolactin group showing greater 5α -reductase activity than did the normal prolactin group. This increase in 5α reduction largely accounted for the higher level of testosterone metabolized by the group of tumors associated with high prolactin.

In contrast, as is shown in Chart 5, there was no difference in the levels of DHA metabolized (as estimated by determining the amounts of DHA remaining unmetabolized) by the same 2 groups of tumors.

DNA Content. Sufficient material was available for DNA estimations on 7 of the tumors from the high prolactin group and 6 from the normal prolactin group. There was no significant difference between the 2 groups, the mean \pm S. E. for the high prolactin group being 8.62 ± 1.14 mg/g tissue and that for the normal prolactin group being 7.89 ± 1.66 mg/g tissue.

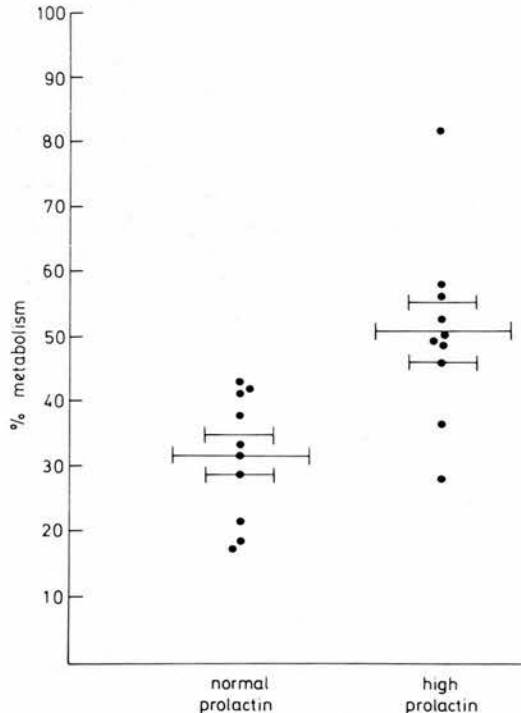


Chart 1. Percentage of metabolism of 7α -[^3H]testosterone by 10 carcinomas induced and grown in the presence of normal prolactin levels compared with 10 carcinomas induced and grown in the presence of high prolactin levels. Lines, mean \pm S. E. for the group; $p < 0.005$.

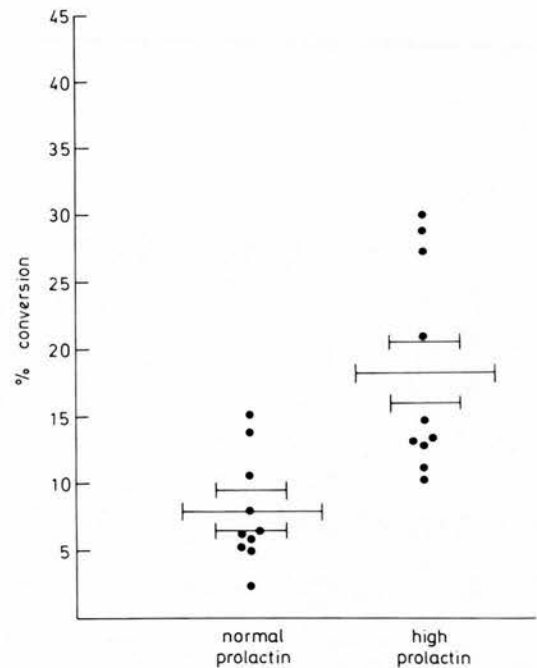


Chart 2. Percentage of conversion of testosterone to 5α -DHT by 10 carcinomas induced and grown in the presence of normal prolactin levels compared with 10 carcinomas induced and grown in the presence of high prolactin levels. Lines, mean \pm S. E. for the group; $p < 0.005$.

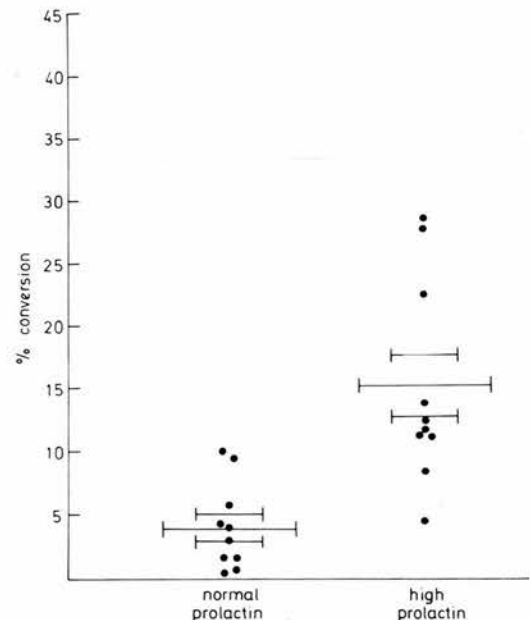


Chart 3. Percentage of conversion of testosterone to 5α -androstanediol by 10 carcinomas induced and grown in the presence of normal prolactin levels compared with 10 carcinomas induced and grown in the presence of high prolactin levels. Lines, mean \pm S. E. for the group; $p < 0.005$.

DISCUSSION

These results indicate that elevation of prolactin during tumor induction and growth gives rise to carcinomas with a greater capacity to metabolize testosterone. The increased

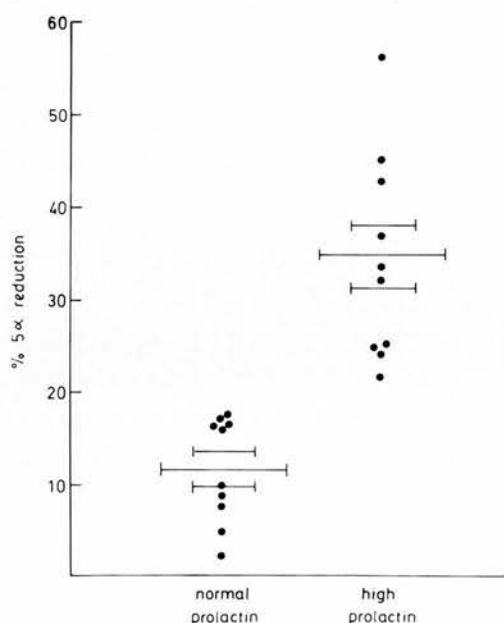


Chart 4. Percentage of 5α reduction of testosterone by 10 carcinomas induced and grown in the presence of normal prolactin levels compared with 10 carcinomas induced and grown in the presence of high prolactin levels. Lines, mean \pm S. E. for the group; $p < 0.001$.

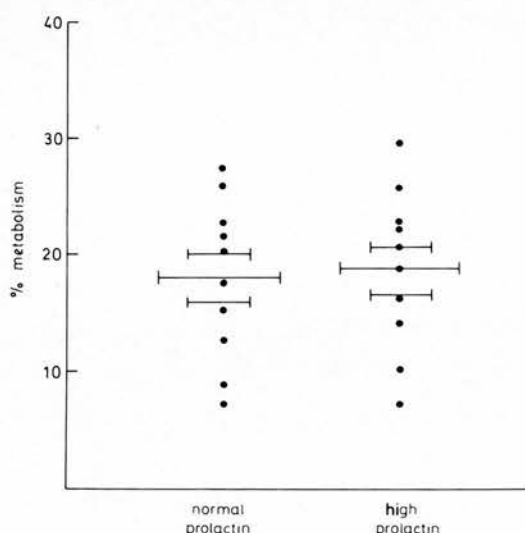


Chart 5. Percentage of metabolism of 7α -[^3H]DHA by 10 carcinomas induced and grown in the presence of normal prolactin levels compared with 10 carcinomas induced and grown in the presence of high prolactin levels. Lines, mean \pm S. E. for the group.

metabolism is largely accounted for by raised 5α -reductase activity, which gives rise to increased production of both 5α -DHT and 5α -androstenediol as compared with the control group. It would seem that these changes in metabolism are relatively specific, as the percentage of transformation of another closely related C_{19} steroid, DHA, was similar in both groups of tumors. This would tend to indicate that the increase in testosterone metabolism of the high prolactin group is not a result of increased cellularity of tumors, a conclusion supported by the similar DNA content of tumors from both groups.

As prolactin levels were raised during both the tumor

induction period and that of tumor growth, it is not possible to indicate whether these changes in tumor metabolism are a result of the induction of tumors with different potential for steroidogenesis or whether the differences in tumor metabolism developed during the growth of the tumors. A study is being carried out in which prolactin levels are elevated only after tumor induction, to elucidate this point.

While prolactin has been shown to influence steroid metabolism in other tissues, such as prostate (2) and testis (6), it is believed that this is the 1st indication that prolactin may affect steroid metabolism in mammary tumors. This finding may assume additional importance, as the 5α -reduced metabolites involved are known to influence tumor growth (7, 8).

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Effects of Prolactin upon C₁₉ Steroid Metabolism by Rat Mammary Carcinoma**WILLIAM R. MILLER, ROBERT BUCHAN and A. P. M. FORREST***Department of Clinical Surgery, Medical School, University of Edinburgh, Edinburgh EH8 9AG, U.K.*

It has been shown that mammary carcinomas induced in female Sprague-Dawley rats by the administration of the carcinogen dimethylbenzanthracene may metabolize steroid hormones (King *et al.*, 1964, 1965). The aim of the present study was to determine the effects of increasing the circulating amounts of prolactin upon the metabolism of dehydroepiandrosterone and testosterone in these tumours.

Two groups of female Sprague-Dawley rats, in whom adenocarcinoma were induced by dimethylbenzanthracene (5mg intravenously at 50 days of age), were investigated. One group received perphenazine (fentazin) in daily subcutaneous injections (5mg/kg body wt.) from the age of 30 days to raise circulating prolactin amounts. The control group received only vehicle (0.2% citric acid). A comparison of tumours believed to be induced and grown in the presence of high and normal amounts of circulating prolactin was therefore possible.

The metabolism of dehydroepiandrosterone and testosterone *in vitro* was determined in ten adenocarcinomas from both groups of animals. A portion (1g) of each tumour was finely sliced in 10ml of Krebs-Ringer phosphate buffer. An NADPH-generating system (200 μ mol of glucose 6-phosphate, 25 μ g of NADP⁺ and 50 units of glucose 6-phosphate dehydrogenase) and 45 Ci of either [7-³H]dehydroepiandrosterone or [7-³H]testosterone were added. Incubation was immediately performed at 37°C for 2h in O₂.

The steroid interconversions were determined by measuring the percentage incorporation of radioactive label into the individual metabolites after extraction and purification by t.l.c. Details of the methods of steroid purification and characterization have been described by Fahmy *et al.* (1968) and Jones *et al.* (1970).

The metabolism of [7-³H]dehydroepiandrosterone was not influenced by perphenazine treatment, no differences being found either in the amount of metabolism of dehydroepiandrosterone or the production of metabolites of dehydroepiandrosterone.

The results from the incubations with testosterone are presented in Table 1. The carcinomas from the perphenazine-treated animals displayed significantly greater metabolism of testosterone than those from control animals. The amount of metabolism varied considerably within each group, the variation being not only between individual animals but between tumours from different sites within the same animal. Of the metabolites of testosterone the mean production of 5 α -dihydrotestosterone was higher (but not significantly so) in tumours from the perphenazine-treated animals. The conversion into 5 α -androstadiol was, however, significantly increased in the perphenazine-treated rats.

Table 1. *Metabolism of testosterone by adenocarcinoma*

Results are given as means \pm s.e.m. for ten tumours, except for * mean for eight tumours and † mean for six tumours. % 5 α -reduction is calculated as combined production of 5 α -dihydrotestosterone and 5 α -androstandiol.

	% testosterone metabolized	% 5 α -dihydrotestosterone produced	% 5 α -androstandiol produced	% 5 α -reduction	% Δ -4-androstenedione produced
Dimethylbenzanthracene alone	34.06 \pm 3.81	10.54 \pm 2.42	4.00 \pm 1.17	14.55 \pm 2.91	0.42 \pm 0.10*
Dimethylbenzanthracene + perphenazine	48.58 \pm 4.33	15.58 \pm 2.27	15.32 \pm 2.43	31.90 \pm 3.97	1.84 \pm 0.37†
Significance	$P = 0.01$	$P = 0.10$	$P < 0.0005$	$P < 0.0025$	$P = 0.05$

By combining the production of 5α -dihydrotestosterone and 5α -androstadiol and expressing the result as % 5α -reduction, an estimate of total 5α -reductase activity was obtained. This was significantly higher in the perphenazine-treated group as compared with the control group.

The conversion of testosterone into Δ -4-androstenedione was also investigated. Although the mean amount of oxidation into Δ -4-androstenedione was significantly higher in the perphenazine group, this was accounted for by two tumours, in which the amount of oxidation was markedly raised. These two tumours had the lowest 5α -reductase activity of the perphenazine group.

It is possible that the increased 5α -reductase activity demonstrated in tumours from perphenazine-treated animals is caused by differences in tumour cellularity. The findings of similar metabolism of another C-19 steroid precursor, dehydroepiandrosterone, and the similar conversion of testosterone into Δ -4-androstenedione in both groups of tumours would, however, indicate that the effects on 5α -reduction of testosterone are relatively specific. Further evidence for this has come from preliminary studies in which ovine prolactin (50 μ g/ml) has been added to incubation mixtures of dimethylbenzanthracene adenocarcinomas *in vitro* with similar effects on testosterone metabolism.

Prolactin has been shown to influence the metabolism of testosterone in other steroid-metabolizing organs. For example, Boyns *et al.* (1972) have demonstrated that prolactin not only increased uptake of testosterone in rat prostatic cultures but changed the ratio of 5α -dihydrotestosterone to testosterone in favour of the non-reduced form. This effect upon 5α -reduction in the prostate is opposite to that demonstrated in the present study of rat mammary tissue.

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The Effect of Perphenazine Treatment on Testosterone Metabolism by Established Rat Mammary Carcinomas

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Mammary carcinomas may be induced in female Sprague-Dawley rats by the administration of the carcinogen 7,12-dimethylbenzanthracene. These tumours metabolize testosterone (King *et al.*, 1964), particularly by 5α -reduction to 5α -dihydrotestosterone and 5α -androstanediol (Miller, 1976). In a previous study, tumours obtained from animals rendered hyperprolactinaemic by giving perphenazine during the period of tumour induction and growth showed enhanced conversion of testosterone into 5α -reduced metabolites (Miller *et al.*, 1974a). The aim of the present study was to determine if similar effects could be elicited by administering perphenazine to animals with established tumours and raising plasma prolactin concentrations during the growth period alone.

Tumours induced in female Sprague-Dawley rats by intragastric administration of dimethylbenzanthracene (30mg in cotton-seed oil) at 50 days of age were measured three times weekly by calipers. Animals bearing tumours showing continuous growth were allocated to control or treatment groups when the tumour size was about 1.5 cm \times 1.5 cm. The treatment group received daily subcutaneous injections of perphenazine (5mg/kg body weight) until being killed 12 days later. Control animals did not receive the drug over the same time-period. Measurement of tumour size continued during treatment until excision at death. The metabolism of testosterone *in vitro* was then determined in ten tumours from each group. A portion of each tumour (0.5 g) was finely sliced and sonicated in 5 ml of Krebs-Ringer phosphate buffer, pH 7.4. An NADPH-generating system (100 μ mol of glucose 6-phosphate, 15 μ mol of NADP⁺ and 25 units of glucose 6-phosphate dehydrogenase) and 20 μ Ci of [7α -³H]testosterone were added to give a final volume of 7.5 ml. The incubations were then shaken in an atmosphere of O₂ at 37°C for 1 h.

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Table 1. *Metabolism of [3H]testosterone by carcinomas from perphenazine-treated and control animals*

Results are given as means \pm s.e.m. for ten tumours, except for * mean for six tumours and † mean for nine tumours. 5 α -Reduction (%) is calculated as combined conversion into 5 α -dihydrotestosterone and 5 α -androstanediol. Significance is calculated by the Wilcoxon Rank test. N.s., Not significant.

	Metabolism of testosterone (%)	Conversion into 5 α -dihydrotestosterone (%)	Conversion into 5 α -androstanediol (%)	5 α -Reduction (%)	DNA content (μ g/mg of tissue)
Control	49.07 \pm 4.59	5.98 \pm 0.98	26.68 \pm 4.47	32.66 \pm 5.07	6.58 \pm 1.42*
Perphenazine-treated	67.65 \pm 3.82	17.28 \pm 3.54	44.66 \pm 2.90	61.94 \pm 5.26	7.93 \pm 0.88†
Significance	$P < 0.01$	$P < 0.05$	$P < 0.01$	$P < 0.01$	N.S.

The steroid interconversions were determined by measuring the percentage incorporation of radioactive label into the individual metabolites after extraction and purification by t.l.c. Details of the methods of steroid purification and characterization have been described by Miller *et al.* (1974b).

When sufficient tumour material was available, the DNA content was measured by the method of Burton (1956).

All but one of the tumours from the perphenazine-treated group showed an acceleration of growth during the treatment period, whereas those from control animals showed no significant change.

The results from the incubations with [7α - ^3H]testosterone are summarized in Table 1. Tumours from perphenazine-treated animals showed significantly higher metabolism of testosterone than those from control animals. The major metabolites of testosterone in tumours from both control and perphenazine-treated animals were 5α -dihydrotestosterone and 5α -androstenediol. The conversion into both 5α -dihydrotestosterone and 5α -androstenediol was, however, significantly higher in tumours from the perphenazine-treated group. The increase in 5α -reduction alone accounts for the higher concentrations of testosterone metabolized by tumours from this group. These effects of perphenazine are unlikely to be mediated by an increase in tumour cellularity, as the tumour DNA content was similar in both groups of animals.

It is concluded that short-term administration of perphenazine stimulates growth and testosterone metabolism in dimethylbenzanthracene-induced rat mammary carcinomas. Preliminary results show that these changes were accompanied by an increase in plasma prolactin.

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Perphenazine and Testosterone Metabolism by Mammary Tumours in Oophorectomized Rats

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Rat mammary tumours induced by the carcinogen 7,12-dimethylbenzanthracene in female rats metabolized testosterone, particularly by 5α -reduction to 5α -dihydrotestosterone and 5α -androstenediol (Miller, 1976). It has also been shown that these conversions were significantly elevated in tumours from animals treated with perphenazine (2-(4-[3-(2-chlorophenothiazin-10-yl)propyl]piperazin-1-yl)ethanol) (Buchan *et al.*, 1976). Additionally, perphenazine stimulated tumour growth, raised plasma prolactin concentrations and interrupted the normal oestrous cycle. To determine the relative influence of prolactin and ovarian hormones on tumour growth and steroid metabolism, the effects of perphenazine have been studied in oophorectomized animals.

Female Sprague-Dawley 50-day-old rats were given 7,12-dimethylbenzanthracene (30mg in cottonseed oil) intragastrically. Tumours subsequently induced were measured three times weekly by calipers throughout the study. Animals bearing tumours that showed continuous growth were allocated to the treatment or the control group when the tumour size exceeded $2\text{cm} \times 2\text{cm}$. The allocation was performed on the day of dioestrus when all animals were bled from the tail vein and bilaterally oophorectomized. The treatment group of rats additionally received daily subcutaneous injection of perphenazine (5mg/kg body wt.) from the day of oophorectomy until death 14 days later. Control animals received daily doses of the injection vehicle (corn oil) over the same period. Immediately before death, blood was taken for plasma prolactin determination by radioimmunoassay, and tumours taken for steroid metabolism studies *in vitro*. Ten tumours from both animal groups were investigated: 0.5g of each tumour was finely sliced and incubated at 37°C for 1h in 5ml of Krebs-Ringer phosphate buffer, pH 7.4, containing $20\mu\text{Ci}$ of $[7\alpha\text{-}^3\text{H}]$ testosterone and NADPH-generating system (Buchan *et al.*, 1976). Methods for purification and characterization of testosterone and its metabolites have been described in detail previously (Miller *et al.*, 1974).

The effects of the treatment regimes on plasma prolactin concentrations are shown in Table 1. Both groups of animals had similar prolactin values at the time of allocation. However, whereas oophorectomy alone resulted in a fall in plasma prolactin concentration in each animal, oophorectomy in combination with perphenazine treatment produced an increase in prolactin in all animals. The difference between pre- and post-treatment values was significantly for both animal groups.

Oophorectomy alone produced an immediate regression of all tumours. In the oophorectomy-plus-perphenazine group, however, there was a variable response in tumour growth, certain tumours increasing in size throughout the time of study whereas others regressed, usually after a short period of continued growth.

The metabolism of $[7\alpha\text{-}^3\text{H}]$ testosterone by tumours from both groups of animals is shown in Table 2. Tumours from animals given perphenazine in addition to oophorect-

Table 1. Effect of oophorectomy and subsequent perphenazine administration on plasma prolactin concentrations

Animal group	Plasma prolactin (ng/ml) (means + S.E.M.)		
	Before treatment	After treatment	Significance*
Oophorectomy alone	37.1 ± 11.4	6.7 ± 1.0	$P < 0.01$
Oophorectomy and perphenazine administration	37.3 ± 10.8	87.9 ± 10.6	$P < 0.01$

* Significance was measured by paired Student's *t* test.

Table 2. *Metabolism of [7 α -³H]testosterone by tumours from the animal groups studied*

Significance, by Wilcoxon-Rank test, of perphenazine-treated animals paired with oophorectomy-alone group for each conversion was: *, not significant; †, $P < 0.1$; ‡, $P < 0.05$.

Animal group	Metabolism of testosterone (%)	Conversion into 5 α -dihydrotestosterone (%)	Conversion into 5 α -androstenediol (%)	5 α -Reduction (%)
Oophorectomy alone	40.09 \pm 5.43	4.53 \pm 0.66	22.96 \pm 3.40	27.49 \pm 3.74
Oophorectomy and subsequent perphenazine administration	57.65 \pm 4.83*	8.97 \pm 2.32†	33.22 \pm 4.43†	42.17 \pm 6.13‡

omy showed significantly higher metabolism of testosterone than tumours from rats subjected to oophorectomy alone. In all tumours, irrespective of the animal group from which they were derived, the major metabolites of testosterone were 5 α -dihydrotestosterone and 5 α -androstenediol. Although the mean conversions into both metabolites were greater in tumours from animals given perphenazine, the differences from the oophorectomy alone-group were not significant.

The present results contrast with effects in intact animals where perphenazine, although producing a similar elevation in plasma prolactin values, stimulates tumour growth and causes a highly significant increase in tumour 5 α -reduction of testosterone (Buchan *et al.*, 1976).

In summary, perphenazine failed to reproduce, in animals deprived of ovarian hormones, the marked stimulatory effects on mammary-tumour growth and steroid metabolism that it produces in endocrine-intact animals.

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Short Communication**HORMONAL STATUS AND TESTOSTERONE METABOLISM OF DMBA-INDUCED RAT MAMMARY CARCINOMAS**

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A MAJOR pathway of steroid metabolism in rat mammary carcinomas is the reduction of testosterone to 5α dihydrotestosterone and 5α androstenediol (King, Gordon and Helfenstein, 1964; Miller, Forrest and Hamilton, 1974). *In vitro* addition of oestradiol 17β to incubations of hormone-dependent rat mammary carcinomas is associated with an inhibition of 5α reduction of testosterone (Miller, 1976a). The aim of the present study was to determine if *in vivo* hormone manipulation was associated with similar changes.

A single i.v. injection of 5 mg 7-12-dimethylbenzanthracene (DMBA) was given to 24 randomly bred female Sprague Dawley rats at 50 days of age. The size of the tumours which were induced was monitored twice weekly by measuring with calipers 2 diameters at right angles. When the tumours were 2×2 cm in size, animals were allocated to one of 3 groups. Those in Group I were killed without further treatment, those in Group II were oophorectomized and killed 14 days later and those in Group III were oophorectomized but 14 days later received daily s.c. injections of $1 \mu\text{g}$ oestradiol in corn oil for a further 14 days, when they were killed. In all animals, oophorectomy led to regression of tumours; all those subsequently given oestradiol showed regrowth.

Tumours were harvested at death and treated at 0°C . Each was finely sliced in Krebs Ringer phosphate buffer pH 7.4 (10 ml/g tumour) and an NADPH-generating system (200 μmol glucose-6-

phosphate, 30 μmol NADP and 50 u glucose-6-phosphate dehydrogenase/g tumour) and $7\alpha^3\text{H}$ -testosterone (45 $\mu\text{Ci/g}$ tumour) added. The incubation systems were then shaken for 1 h at 37°C in an atmosphere of O_2 . The reaction was stopped by adding methanol to 80% v/v and the incubations stored at -10°C until processed.

Before extraction, 500 μg of non-radioactive carrier testosterone (17β -hydroxy-4-androstene-3-one), 5α dihydrotestosterone (17β -hydroxy- 5α -androstane-3-one) from Sigma Chemical Co. (St Louis, Mo.) and 5α androstenediol (5α -androstane 3β 17β diol) from Steraloids Inc. (N.Y.) were added to monitor recovery losses. The metabolites were extracted, separated into individual steroids and purified by thin layer chromatography as described previously (Miller *et al.*, 1974). The metabolism of testosterone by conversion to 5α dihydrotestosterone and 5α androstenediol were determined by measuring the percentage incorporation of radioactive label into the appropriate metabolites after correction for recovery losses.

The DNA content of the tumours was determined by a modification of the method of Burton (1956).

The pattern of testosterone metabolism by tumours from the 3 groups of animals is shown in Table I. There was an increased metabolism of testosterone in tumours harvested 14 days after oophorectomy, an effect reversed by administration of oestradiol 17β . Tumours from oestrogen-treated animals showed signifi-

TABLE I.—*Endocrine Status and Tumour Metabolism of 7 α ³H-testosterone*

Endocrine status of animal	% Testosterone metabolized Mean \pm s.e. (range)	% Conversion to	
		5 α Dihydrotestosterone Mean \pm s.e. (range)	5 α Androstenediol Mean \pm s.e. (range)
1. Intact*	53.10 \pm 5.05 (34.5–74.5)	15.50 \pm 3.90 (4.4–30.7)	13.95 \pm 1.50 (8.4–20.3)
2. Oophorectomized**	66.50 \pm 5.25 (53.5–88.0)	15.40 \pm 2.70 (5.5–30.1)	27.00 \pm 3.70 (16.4–49.1)
3. Oophorectomized*** + oestradiol	34.45 \pm 4.20 (16.8–51.5)	9.20 \pm 1.90 (3.6–19.2)	14.20 \pm 2.45 (7.3–28.10)
† 1 v 2	$P < 0.10$	Not significant	$P < 0.02$
2 v 3	$P < 0.01$	Not significant	$P < 0.01$
1 v 3	$P < 0.05$	Not significant	Not significant

*Tumours from hormonally unmanipulated animals.

**Tumours from animals 14 days after oophorectomy.

***Tumours from animals oophorectomized but 14 days later given oestradiol (1 μ g in corn oil) for a further 14 days.

†Significance between groups by Wilcoxon rank tests.

cantly less metabolism of testosterone than those from either intact or oophorectomized groups.

Although 5 α dihydrotestosterone was a metabolite of testosterone in all tumours, there was no significant difference in its production between the animal groups, despite a lowered mean production in the group receiving oestrogen. In contrast, the conversion of testosterone to its other major 5 α reduced product, 5 α androstenediol, was clearly influenced by hormone manipulation, and was significantly increased in tumours from oophorectomized rats. The subsequent administration of oestradiol was associated with a significant decrease in production of 5 α androstenediol, to levels comparable with tumours from the intact group.

Sufficient material was available for determination of DNA content in 6 tumours from each group. No significant differences were observed (Table II).

TABLE II.—*DNA Content of Tumours Studied*

Endocrine status of animal	DNA content (mg/g tumour \pm s.e.)
Intact (6)	7.00 \pm 0.74
Oophorectomized (6)	7.69 \pm 0.79
Oophorectomized + oestradiol (6)	7.66 \pm 2.03

Figures in parenthesis are number of tumours studied. No significant differences between the groups by Wilcoxon-rank test.

These results indicate that, in female Sprague Dawley rats bearing DMBA-induced mammary carcinomas, oophorectomy is associated with an increase in tumour metabolism of testosterone, a phenomenon which may be reversed by *in vivo* administration of oestradiol. A similar pattern was observed in the conversion of testosterone to 5 α androstenediol. Increased production of 5 α androstenediol in tumours from oophorectomized animals alone would account for the higher levels of testosterone metabolized by these tumours. In contrast, reduced formation of 5 α androstenediol does not fully account for the decreased levels of testosterone metabolized in tumours from oestrogen-treated animals: reduced conversion to 5 α dihydrotestosterone in these tumours also contributes to the decreased metabolism of testosterone.

As the DNA content of tumours from each animal group was similar, these changes are unlikely to be caused by differences in tumour cellularity. Furthermore, the decreased metabolism and conversion of testosterone to 5 α androstenediol following *in vivo* administration of oestrogen may be reproduced *in vitro* by addition of oestradiol to incubations of hormone-dependent rat mammary carcinomas (Miller, 1976a).

Because the methods used in these studies estimate the total production of

all 4 isomers of 5α androstenediol (Miller, 1976a), it is not possible to determine if the production of a particular isomer is preferentially affected by hormone manipulation. However, only 17β isomers of androstenediol were identified as metabolites of testosterone in human breast cancer (Cameron *et al.*, 1971).

It is possible that oophorectomy and oestrogen administration affect tumour metabolism indirectly, by respectively lowering and raising circulating levels of prolactin, to which the growth of DMBA-induced tumours are particularly sensitive (Meites, 1972; Pearson *et al.*, 1972). Nevertheless the *in vitro* addition of oestradiol (Miller, 1976a), as well as prolactin (Miller, 1976b), has been shown to influence testosterone metabolism by DMBA rat tumours and both hormones may therefore be implicated in the changes effected by the endocrine manipulations described in this study.

Whether the effects of oestrogen administration are caused directly by oestradiol 17β , or indirectly by prolactin secretion, or both, the lower synthesis of 5α dihydrotestosterone and 5α androstenediol in growing tumours from animals given oestrogen, as compared with regressing tumours after oophorectomy, is in keeping with the growth-inhibiting properties of 5α reduced steroids (Huggins and Mainzer, 1957). However, without further work to determine the sequence of events following hormone manipulation, it is not possible to indicate whether changes in steroid metabolism occur before or concurrently with those in tumour growth, or whether the change

in tumour growth itself leads to differences in metabolism of testosterone.

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Short Communication

IN VITRO EFFECTS OF OESTROGEN ON 5 α -REDUCTION OF TESTOSTERONE IN HORMONE-DEPENDENT RAT MAMMARY CARCINOMATA

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FEMALE Sprague Dawley rats given 7-12-dimethylbenzanthracene (DMBA) develop mammary carcinomata, most of which are hormone-dependent, regressing following oophorectomy but regrowing after oestrogen administration (Huggins, 1963). In these tumours, regression may also be produced by administration of 5 α -reduced steroids (Huggins, Briziarelli and Sutton, 1959; Huggins and Mainzer, 1957). It is therefore of interest that DMBA-induced rat mammary tumours have the potential to synthesize 5 α -reduced steroids (King, Gordon and Helfenstein, 1964; Miller, Forrest and Hamilton, 1974). The aim of the present study was to determine the effects of oestrogen on tumour 5 α -reduction of testosterone.

Tumours were induced in randomly bred female Sprague Dawley rats by intravenous administration of 5 mg DMBA at 50 days of age. When the tumours were approximately 2 \times 2 cm in size the rats were oophorectomized. Fourteen days after oophorectomy the animals were given daily subcutaneous injections of oestradiol-17 β in corn oil (1 μ g or 5 μ g). This regime was continued for a further 14 days when the animals were sacrificed by exsanguination. No injection was given on the day of sacrifice. Tumour size was monitored throughout the study by measuring with calipers the two major diameters at right angles, and expressing the size of the resulting multiple in cm². Measurement was performed twice weekly

until oophorectomy and three times weekly thereafter. Only tumours which showed consistent regression after oophorectomy and regrowth with oestrogen treatment were classified as hormone-dependent and taken for incubation.

All tumours were processed at 0°C until incubation (within 30 min of tissue removal). The tumours were finely sliced and split into duplicate portions each weighing 1 g. Krebs-Ringer phosphate buffer pH 7.4 (10 ml), an NADPH-generating system (200 μ mol glucose-6-phosphate, 30 μ mol NADP and 50 units glucose-6-phosphate dehydrogenase) and 45 μ Ci 7 α -³H testosterone (sp. act. 12.4 Ci/mmol from Radiochemical Centre, Amersham) were added to each. One incubation mixture was used without further addition as a control; to the other was added oestradiol-17 β (1.5 μ g/ml) to determine the effects of oestrogen. Both systems were then incubated by shaking at 37°C in an atmosphere of oxygen for 1 h. The reaction was stopped by adding methanol (60 ml) and the incubations were stored at -10°C until the steroids were isolated and characterized.

Before extraction, 500 μ g non-radioactive carrier steroids (testosterone (17 β -hydroxy-4-androsten-3-one), 5 α dihydrotestosterone (17 β -hydroxy-5 α -androsten-3-one) and 5 α androstenediol (5 α -androstane 3 β 17 β diol)) were added to monitor recovery losses. The metabolites were extracted as described by Fahmy *et al.*

(1968) and separated into individual steroids by continuous elution thin layer chromatography for 2 h on Silica gel HF254+366 in chloroform:acetone (98:2). Purification of testosterone and 5 α dihydrotestosterone involved sequential acetylation and hydrolysis; that for 5 α androstenediol sequential oxidation and reduction (derivative formation and chromatography systems as in Miller *et al.*, 1974). Although 5 α androstenediol was added as the 3 β 17 β isomer, the methods described estimate total production of all 4 isomers of 5 α androstenediol since the isomers migrate together in the initial chromatography system and the subsequent oxidation step yields a common product, 5 α androstenedione. The percentage metabolism of testosterone and conversion to 5 α dihydrotestosterone (DHT) and 5 α androstenediol were determined by measuring the percentage incorporation of radioactive label into the appropriate metabolites after correction for recovery losses. Total 5 α -reduction

was calculated by combining the percentage production of both 5 α DHT and 5 α androstenediol.

The results from these incubations are presented in Table I. There was a wide variation in metabolism of testosterone between individual tumours. *In vitro* addition of oestradiol produced variable results on the level of testosterone metabolized, although the most common effect was one of inhibition. Of the two 5 α -reduced metabolites of testosterone, the production of 5 α androstenediol usually exceeded that of 5 α DHT, in incubations without added oestradiol. The *in vitro* addition of oestradiol produced variable effects on the production of 5 α DHT, although in tumours with the highest control production of 5 α DHT, oestradiol was consistently inhibitory. Oestradiol inhibited the production of 5 α androstenediol in all carcinomata, with a single exception, a tumour in which oestradiol exclusively affected the production of 5 α DHT. This meant that total

TABLE I.—*In vitro* Effects of Oestradiol on Steroid Metabolism by 10 Hormone-dependent Rat Mammary Carcinomata

Tumour	% Testosterone metabolized	% 5 α DHT produced	% 5 α Androstenediol produced	% 5 α -reduction
1.* Control	16.85	5.85	9.60	15.45
Treated	25.25	4.50	0.80	5.30 (–66)
2.* Control	51.50	9.20	28.05	37.25
Treated	37.05	12.15	1.75	13.90 (–63)
3.* Control	87.30	7.00	38.20	45.20
Treated	71.30	9.10	24.00	33.10 (–25)
4.* Control	38.30	12.55	23.05	35.41
Treated	36.20	8.75	15.45	24.20 (–32)
5.* Control	34.70	6.25	14.25	20.50
Treated	26.90	4.55	11.05	15.60 (–24)
6.† Control	89.10	22.75	37.20	59.95
Treated	77.15	7.85	39.95	47.80 (–20)
7.† Control	63.90	36.10	27.05	63.45
Treated	40.05	14.15	22.20	36.35 (–43)
8.† Control	38.35	9.80	28.45	38.25
Treated	37.35	8.95	20.30	29.25 (–24)
9.† Control	73.35	9.95	33.10	43.05
Treated	69.25	8.00	25.09	33.05 (–23)
10.† Control	74.45	37.70	35.80	73.50
Treated	69.10	26.65	25.50	52.15 (–29)

Control: Tumour incubated without oestradiol.

Treated: Tumour incubated in the presence of 1.5 μ g/ml oestradiol.

Figures in parentheses represent percentage change produced by addition of oestradiol.

* Tumour regrowth following administration of oestradiol (1 μ g/day).

† Tumour regrowth following administration of oestradiol (5 μ g/day).

TABLE II.—*In vitro* Effects of Oestradiol on Steroid Metabolism by Hormone-independent Rat Mammary Carcinomata

	Tumour	% Testosterone metabolized	% 5 α DHT produced	% 5 α Androstenediol produced	% 5 α -reduction
1.*	Control	72.10	14.49	7.26	21.75
	Treated	58.97	12.44	7.50	19.94 (–8)
2.†	Control	89.27	42.72	22.17	64.89
	Treated	92.35	41.61	45.64	87.25 (+34)

Control: Tumour incubated without oestradiol.

Treated: Tumour incubated in the presence of 1.5 μ g/ml oestradiol.

Figures in parentheses represent percentage change produced by addition of oestradiol.

* Tumour growth continuous after both oophorectomy and administration of oestradiol (1 μ g/day).

† Tumour growth continuous after oophorectomy but stimulated by administration of oestradiol (1 μ g/day).

5 α -reduction was inhibited by oestradiol in all tumours, the level of inhibition varying between 20 and 65%.

Whilst in some tumours inhibition of 5 α -reduction alone would account for the effects of oestradiol on percentage metabolism of testosterone, in certain tumours, oestradiol must have also affected other steroid conversions. The production of Δ 4 androstenedione and 5 α androstenedione was also investigated in several tumours, but never exceeded 1% and did not appear to be influenced by *in vitro* addition of oestradiol.

These results indicate that oestradiol 17 β may influence steroid metabolism by rat mammary carcinomata. *In vitro* addition of oestradiol reduces tumour synthesis of 5 α -reduced metabolites from testosterone, particularly 5 α androstenediol.

Although this is the first report that oestrogen may affect the production of 5 α -reduced steroids by mammary cancers, it is well documented that 5 α -reduction may be hormonally controlled in other tissues such as liver (Schriefers, 1967), adrenal cortex (Kitay, Coyne and Swygert, 1970) and prostate (Farnsworth, 1972). In common with the results presented in this study for mammary tissue, oestradiol inhibits 5 α -reduction in both adrenal cortex and prostate.

The synthesis of 5 α -reduced steroids assumes added importance in mammary tumours because both 5 α DHT and 5 α androstenediol inhibit the growth of the

hormone-dependent rat mammary tumour (Huggins *et al.*, 1959; Huggins and Mainzer, 1957). These effects of oestradiol in decreasing tumour synthesis of 5 α -reduced steroids would therefore be in keeping with oestradiol's growth-promoting effects in hormone-dependent tumours. In this context it is interesting that the same concentration of oestradiol failed to inhibit 5 α -reduction in two hormone-independent rat mammary carcinomata (Table II). Further numbers are required before it will be possible to determine if this represents a distinction between hormone-dependent and hormone-independent tumours.

Although the level of oestradiol added *in vitro* (1.5 μ g/ml) is high compared with normal plasma levels in female rats (0.1–4.4 ng/100 ml, Hawkins *et al.*, 1975), the dose used in this study is comparable with that which *in vitro* inhibits 5 α -reduction of testosterone in prostatic tissue (Griffiths *et al.*, 1970; Jenkins and McCafferty, 1974) and that used in predicting oestrogen sensitivity in human breast tumours (Salih, Flax and Hobbs, 1972).

It remains to be seen, however, if oestradiol *in vivo* has similar effects on tumour steroidogenesis. Although oophorectomy increases the level of 5 α -reduction in rat mammary carcinomata, an effect which can be reversed by administration of oestrogen (Miller *et al.*, 1974), this could be caused by changes in circulating oestrogen or prolactin.

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General Papers

In Vitro Effects of Prolactin upon Testosterone Metabolism by Rat Mammary Adenocarcinomata*

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Abstract—The effects of *in vitro* addition of prolactin (50 µg/ml) upon the conversion of testosterone to 5α dihydrotestosterone (5α DHT) and 5α androstenediol by incubates of rat mammary carcinomas have been investigated. The studies have been performed on (a) primary adenocarcinomata induced by the carcinogen 7,12-dimethylbenzanthracene (DMBA) in female Sprague-Dawley rats, and (b) transplantable tumours obtained by serial transplantation of a further DMBA-induced tumour into neonatally thymectomized rats. In incubations of primary DMBA-induced tumours, addition of prolactin inhibited the production of both 5α DHT and 5α androstenediol, the overall 5α reduction being between 27 and 40% lower than that in control incubations. In contrast, prolactin failed to inhibit, and in some cases stimulated, the 5α reduction of testosterone in the transplantable tumour.

INTRODUCTION

BOTH PROLACTIN and steroid hormones are known to influence the growth and development of rat mammary tumours [1-3]. In the DMBA treated rat, there appears to be a direct correlation between plasma prolactin levels and the genetically determined susceptibility to mammary cancer [4]. Elevation of plasma prolactin levels accelerates the growth of mammary tumours [5-7] and conversely lowered levels may cause tumour regression [8]. Similarly, administration of steroids of the androstane series such as 5α dihydrotestosterone may inhibit the growth of mammary tumours in the rat [9, 10].

The aim of the present study was to determine the *in vitro* effects of prolactin, upon the production of 5α androstane steroids by rat mammary tumours.

MATERIAL AND METHODS

Tumour tissue

(a) *DMBA-induced tumours.* Random bred female Sprague-Dawley rats were given 5 mg dimethylbenzanthracene (DMBA) intravenously at 50 days of age. Subsequently induced mammary tumours were allowed to grow to 2 × 2 cm in size. At this stage, three rats were sacrificed by exsanguination and the tumours incubated; three others were sequentially oophorectomised and given oestradiol-17β (1 µg) to determine tumour hormone dependence. All tumours regressed after oophorectomy but renewed growth following oestrogen administration and were therefore hormone dependent.

(b) *Transplanted tumours (TG 3).* The tumour studied was originally induced by DMBA as above, in an inbred Sprague-Dawley rat (ADRA) [4]. It was serially transplanted into further ADRA rats (neonatally thymectomized) by dorsal skin implantation and studied at its 2nd and 6th passages. Although all the tumours investigated were derived from intact rats, the

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same tumour at its 6th passage in other rats, failed to regress following oophorectomy but was stimulated by oestradiol (1 μ g daily). Tumour growth was therefore independent of oophorectomy but sensitive to oestradiol.

Tumour processing and incubation

All tumours were processed at 0°C until incubation was carried out (within 30 min of tissue removal). One gram of each tumour was finely sliced and Krebs Ringer phosphate buffer pH 7.4 (10 ml), an NADPH generating system (200 μ mol glucose-6-phosphate, 25 mg NADP, and 50 units glucose-6-phosphate dehydrogenase) and the radioactive precursor (45 μ Ci 7 α -³H testosterone) added. Duplicate incubations were set up with the addition of either ovine prolactin, rat prolactin or rat growth hormone (50 μ g/ml). The tumour slices were then incubated by shaking at 37°C in an atmosphere of oxygen for 1 hr. The reaction was stopped by adding methanol to 80% (v/v) and the incubations stored at -10°C until processed.

Purification and characterization of metabolites

Before extraction, 500 μ g of non-radioactive carrier steroids were added to monitor recovery losses. The metabolites were extracted, separated into individual steroids and purified by thin layer chromatography, as described previously [11]. The percentage of metabolism of testosterone and conversion to 5 α DHT and 5 α androstanediol were determined by measuring the percentage of incorporation of radioactive label into the appropriate metabolites after correction for recovery losses.

RESULTS

The effects on testosterone metabolism of *in vitro* addition of prolactin to incubations of DMBA-induced tumours are presented in Table 1. No consistent effects were produced by prolactin on the absolute level of testosterone metabolised. Prolactin, however, inhibited the conversion of testosterone to both 5 α DHT and 5 α androstanediol. Estimates of total 5 α reduction obtained by combining the production of 5 α DHT with that of 5 α androstanediol showed that prolactin produced a consistent inhibition of 5 α reduction (27.4–40.3% of control incubations), irrespective of the type of prolactin used, or whether the tumour was obtained from intact, or endocrine manipulated rats. Similar *in vitro* addition of rat growth

hormone at the same concentration had negligible effects in 4 tumours and inhibited 5 α reduction in only one tumour (to a lesser extent than prolactin).

The results of similar studies using the transplanted tumour are presented in Table 2. In these incubations, the overall level of testosterone metabolism was extremely high and relatively unaffected by the addition of prolactin. In contrast to the primary DMBA tumours, in this transplanted tumour, prolactin tended to increase the percentage of production of 5 α DHT and total 5 α reduction of testosterone.

DISCUSSION

The results presented in this study indicate that the 5 α reduction of testosterone by rat mammary carcinomas may be influenced by the *in vitro* addition of either rat or ovine prolactin. These effects may be partially specific to prolactin as the *in vitro* addition of growth hormone at the same concentration had less or no effect on testosterone metabolism.

In primary DMBA-induced tumours, prolactin consistently inhibited the production of 5 α reduced metabolites of testosterone. This is interesting in view of the inhibitory properties of the 5 α androstane steroids on the growth of hormone dependent rat mammary tumours [9, 10]. Although evidence for hormone dependence was obtained for only three of the DMBA tumours studied in this series, it was our experience that the majority of DMBA tumours induced in this group of animals were hormone dependent. It would therefore be in keeping with the growth promoting effects of prolactin on hormone dependent tumours that prolactin should inhibit the production of 5 α reduced steroids at tumour level.

The results from *in vitro* incubations of the transplanted tumour differ from the primary DMBA tumours in two major respects. Firstly, although the DNA content and cellularity of the two groups of tumours were similar, the overall metabolism and 5 α reduction of testosterone in control incubations was, in general, higher in the transplanted tumour. Secondly, prolactin tended to stimulate 5 α reduction in the transplanted tumour in direct contrast to its inhibitory effects in the primary tumours. Several factors may be implicated in these differences between transplanted and DMBA tumours. Firstly, whereas the DMBA tumours are likely to be hormone dependent, the transplanted tumour failed to regress following

Table 1. Metabolism of 7α - 3H testosterone by DMBA induced tumours

Rat		Per cent testosterone metabolised	Per cent conversion to 5α DHT	Per cent conversion to 5α androstenediol	Per cent 5α reduction
A*	Control	94.80	28.50	25.40	53.90
	+ prolactin (rat)	85.00 (-10.3)	13.20 (-53.6)	21.30 (-16.1)	34.50 (-36.0)
	+ growth hormone	90.20 (-4.9)	24.20 (-15.1)	22.10 (-13.0)	46.30 (-14.1)
B†	Control	46.50	5.50	27.05	32.55
	+ prolactin (rat)	35.10 (-24.5)	3.90 (-29.1)	17.00 (-37.2)	20.90 (-35.8)
	+ growth hormone	46.10 (-0.9)	6.20 (+12.7)	26.70 (-1.3)	32.90 (+1.1)
C*	Control	47.45	16.15	14.15	30.30
	+ prolactin (rat)	43.10 (-9.2)	8.40 (-49.0)	13.55 (-4.2)	21.95 (-27.6)
	+ growth hormone	48.20 (+1.6)	13.60 (-15.8)	17.20 (+21.55)	30.80 (+1.7)
D†	Control	42.50	5.05	20.10	25.15
	+ prolactin (rat)	37.10 (-12.6)	2.50 (-50.5)	13.30 (-33.8)	15.80 (-37.2)
E*	Control	33.85	5.65	12.45	18.10
	+ prolactin (ovine)	34.75 (+2.6)	5.50 (-2.7)	7.60 (-39.0)	13.10 (-27.6)
	+ growth hormone	31.60 (-6.7)	5.00 (-11.5)	11.55 (-7.2)	16.55 (-8.6)
F†	Control	47.80	19.20	11.20	30.40
	+ prolactin (ovine)	59.40 (+24.3)	9.40 (-51.1)	8.75 (-21.9)	18.15 (-40.3)
	+ growth hormone	44.45 (-7.0)	19.40 (+1.0)	3.95 (-64.7)	23.35 (-23.2)

Figures in parentheses represent the percentage of change caused by addition of prolactin or growth hormone (50 μ g/ml).

*Intact rat.

†Rat sequentially oophorectomized and given oestradiol (1 μ g daily).

Table 2. Metabolism of 7α - 3H testosterone by transplanted tumours

Rat		Per cent testosterone metabolised	Per cent conversion to 5α DHT	Per cent conversion to 5α androstenediol	Per cent 5α reduction
1*	Control	88.70	19.90	31.30	51.20
	+ prolactin (ovine)	95.50 (+7.7)	26.50 (+33.2)	59.00 (+88.5)	85.50 (+67.0)
2*	Control	96.80	9.65	33.60	43.25
	+ prolactin (ovine)	97.15 (+0.3)	21.90 (+127.0)	30.30 (-9.8)	52.20 (+20.7)
3*	Control	98.70	13.30	66.15	79.45
	+ prolactin (ovine)	95.50 (-3.3)	16.50 (+24.0)	66.10 (0)	82.60 (+4.0)
4†	Control	94.00	9.50	39.20	48.70
	+ prolactin (rat)	97.00 (+3.1)	24.50 (+158.0)	31.70 (-19.1)	56.20 (+15.4)
5†	Control	96.70	3.40	50.70	54.10
	+ prolactin (rat)	88.05 (-9.0)	5.25 (+54.4)	55.10 (+8.6)	60.35 (+11.6)

*Tumour at 2nd passage.

†Tumour at 6th passage.

Figures in parentheses represent % change caused by addition of prolactin.

oophorectomy; the effects of prolactin may therefore reflect differences between hormone dependent and hormone sensitive tumours. Secondly, there are different circulating prolactin levels in the two strains of rats used—the ADRA strain, in which the transplanted tumour was induced and subsequently transplanted, has a lower basal level of circulating prolactin, in comparison with the random-

bred variety in which the primary DMBA tumours were induced [4].

At present, it is not possible to remove the strain difference as tumour induction rate by DMBA in the ADRA rat is exceptionally low and the transplanted tumour is not accepted by randomly bred animals.

In conclusion, it is worth emphasizing that whilst prolactin has already been shown to

affect steroid metabolism in other tissues, such as the prostate [12] and testis [13], its effects in mammary tumour tissue may assume added importance in view of the properties of the 5 α reduced metabolites involved to influence tumour growth.

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THE EFFECT OF OESTROGEN ON STEROID METABOLISM BY RAT MAMMARY CARCINOMAS

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Human breast cancer can act as a paraendocrine organ by transforming steroid precursors into hormones with powerful biological activity (Adams and Wong ¹, Miller and Forrest ²). Thus breast cancer has the potential to transform dehydroepiandrosterone sulphate which circulates in large amounts in the plasma of both pre- and postmenopausal women into the active androgens, 5 α dihydrotestosterone (5 α DHT) and 5 α androstenediol. The active oestrogen, oestradiol 17 β , may also be synthesised by a similar pathway. A summary of our findings on steroid metabolism by human breast cancers is presented in table I. All tumours converted dehydroepiandrosterone to testosterone and testosterone to 5 α DHT and 5 α androstenediol but only a proportion of carcinomas could synthesise oestradiol 17 β .

The aims of these studies are twofold: firstly to determine if steroid metabolism differs in hormone dependent and hormone independent tumours. Secondly to determine in these two types of tumour the effects of oestradiol on tumour steroidogenesis, especially 5 α reduction. Work is in progress, studying human breast cancers, but the hormone depen-

dence of these human tumours is not yet known. Parallel studies have therefore been performed in experimental animal tumours whose hormone dependence is already defined. It is this work using rat mammary tumours which is the subject of this paper.

Mammary tumours induced in female Sprague Dawley rats may be classified as hormone dependent or hormone independent according to their response to oophorectomy. Hormone dependent tumours regress following oophorectomy but regrow on administration of daily injections of oestradiol 17 β . In contrast hormone independent tumours continue to grow even after oophorectomy.

Using this differential response after oophorectomy as the criterion of hormone dependence, steroid metabolism in five hormone dependent rat mammary carcinomas has been compared with that in two hormone independent tumours.

Each tumour was finely sliced and split into duplicate portions both weighing one gram. The slices were then suspended in Krebs Ringer phosphate buffer and 50 μ Ci of H³ testosterone added. One system was used without

TABLE I. — *Steroid conversions by human breast cancer.*

Precursor	Metabolite	Total tumours investigated	Tumours with conversion
Dehydroepiandrosterone	Testosterone	9	9
Testosterone	5 α Dihydrotestosterone	25	25
	5 α Androstenediol	25	25
	Oestradiol 17 β	24	12

further addition as a control incubation; to the other, α estradiol was added at a concentration of 1.5 μ g/ml to determine the effects of α -trogen. Both systems were then incubated for 1 hour at 37°C in an atmosphere of oxygen.

The pattern of steroid metabolism was determined by measuring the percentage incorporation of ^3H into the metabolites after purification and characterization. There are two major 5 α reduced products of testosterone (5 α dihydrotestosterone and 5 α androstane-diol). Total 5 α reduction was therefore determined by combining the % production of both metabolites.

The results obtained from these incubations are presented in table II. Regarding the overall testosterone metabolism in the control incubations, there was a wide variation in metabolism between individual tumours ranging from 20 to 90 % transformation. Nevertheless the two hormone independent tumours were

amongst those with the highest metabolism. *In vitro* addition of α estradiol 17 β to the incubations produced variable results on the level of testosterone metabolised, although the most common effect was that of inhibition.

There was no difference between hormone dependent and hormone independent tumours in total 5 α reduction of control incubations. However, the effects of α estradiol on 5 α reduction were different in the two types of tumour. Thus, in each of the hormone dependent tumours, the incubation containing α estradiol showed consistently less 5 α reduction than the corresponding control incubation.

The level of inhibition of 5 α reduction by α estradiol was between 25 and 65 % of the control value. In contrast α estradiol did not inhibit 5 α reduction in either of the hormone independent tumours, having no effect in one tumour and stimulating 5 α reduction in the other.

The inhibitory effects of α estradiol on 5 α reduction in hormone dependent tumours is interesting, as 5 α reduced products powerfully inhibit the growth of the hormone dependent rat mammary tumour (Huggins and Mainzer ²). The effects of α estradiol in decreasing tumour synthesis of 5 α reduced steroids are therefore in keeping with the growth promoting effects of α estradiol in hormone dependent tumours.

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SUMMARY

In summary, it can be concluded that: a) the effects of α estradiol on 5 α reduction may differ

TABLE II. — Metabolism of 7 α ^3H testosterone by rat mammary carcinomas.

	% testosterone metabolised	% 5 α reduction	
<i>Dependent tumours.</i>			
1. Control	16.25	15.45	
+ OE2	25.25	5.30	(— 66 %)
2. Control	51.50	37.25	
+ OE2	37.05	13.95	(— 63 %)
3. Control	38.30	35.60	
+ OE2	36.20	24.15	(— 32 %)
4. Control	63.90	63.40	
+ OE2	40.05	36.35	(— 43 %)
5. Control	73.35	43.05	
+ OE2	69.25	33.05	(— 23 %)
<i>Independent tumours.</i>			
1. Control	72.10	21.75	
+ OE2	58.95	19.95	(— 8 %)
2. Control	89.29	64.90	
+ OE2	92.35	87.25	(+ 24 %)

Control = tumour incubated without oestradiol 17 β
+ OE2 = tumour incubated in the presence of oestradiol 17 β 1.5 μ g/ml

Figures in parenthesis represent % change produced by addition of oestradiol 17 β

fer in hormone dependent and hormone independent tumours; b) oestradiol may promote the growth of hormone dependent tumours by reducing tumour levels of 5α reduced steroids. It remains to be seen if oestradiol has similar effects in human breast tumours.

• • •

a) Les effets de l'oestradiol sur la 5α réduc-

tion sont différents pour les tumeurs hormonodépendantes et non.

b) L'oestradiol peut stimuler la croissance de tumeurs hormonodépendantes en réduisant, dans la tumeur, la teneur en stéroïdes 5α réduits.

c) L'application de ces données au cancer du sein dans l'espèce humaine n'est pas encore démontrée.

EFFECTS OF SERIAL PASSAGE ON THE ENDOCRINE RESPONSE AND STEROID METABOLISM OF A RAT MAMMARY CARCINOMA

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Summary.—A rat mammary carcinoma induced by 7-12 dimethylbenzanthracene was serially transplanted into successive generations of thymectomized host animals. After its 2nd passage, the growth of the tumour appeared hormone-dependent, regressing after oophorectomy and regrowing with administration of oestradiol 17β to the host. Third-generation transplanted tumours, however, showed only a transient regression after oophorectomy, and the growth of tumours after further passages appeared ovary-independent. Loss in hormone dependency was not accompanied by histological changes. There was however a progressive increase with successive transplantation in the ability of tumours to metabolize 7α [^3H] testosterone *in vitro*. This was accounted for by raised conversion to 5α androstenediol.

RAT MAMMARY TUMOURS induced by the carcinogen 7-12 dimethylbenzanthracene (DMBA) have been shown to metabolize testosterone, primarily by 5α reduction, an activity which may be influenced by hormones both *in vitro* (Miller, 1976a) and *in vivo* (Miller, 1976b,c; Buchan *et al.*, 1976). The growth of most DMBA-induced tumours is hormone-dependent. In the study reported in this paper a rat mammary tumour, originally induced by DMBA, has been transplanted into successive generations of host animals. Effects of transplantation on hormone dependence and tumour metabolism of testosterone have been investigated.

MATERIALS AND METHODS

Animals.—All animals used were of an inbred strain of Sprague-Dawley rat, obtained from the Animal Diseases Research Association (ADRA), Moredun Institute, Edinburgh.

Tumour line.—The line (TG5) was derived from a mammary tumour induced in a female ADRA rat by i.v. administration of 5 mg DMBA at 50 days of age. A portion of this primary tumour was cut into 1mm cubes in

Hartmann-Ringer lactate solution. These were aspirated into a narrow-bore cannula using a syringe and then implanted through a small skin incision on to the back of neonatally thymectomized host animals. Once established, these tumour transplants were classified TG 5/1. Successive generations of tumour were transplanted in the same way and classified TG 5/n, where n represents the number of passages.

Experimental protocol.—Except for TG 5/1, which was established in only a single animal, each tumour generation was studied in 4 animals. Two animals were killed without having received endocrine manipulation, and the tumours removed.

The remaining 2 rats were bilaterally oophorectomized and 10 days later given daily injections of oestradiol 17β (1 μg in 0.2 ml corn oil) for a further 10 days, when the animals were killed and the tumours removed. Tumours were measured with calipers on alternate days from when palpable until death. Size was expressed as the product of 2 diameters at right angles in cm^2 .

Tumour steroid metabolism.—A portion of each tumour (0.5 g) was finely sliced in Krebs-Ringer phosphate buffer, pH 7.4 (5 ml). An NADPH-generating system and 20 μCi [7α ^3H] testosterone was added and the systems incubated for 1 h at 37°C in an

atmosphere of O_2 . Reaction was halted by addition of methanol (30 ml) and the incubations stored at $-10^\circ C$ until the metabolites were characterized by the methods previously described (Miller *et al.*, 1974). Metabolism and conversion of testosterone were determined by measuring radioactive label in the appropriate metabolites. Estimation of 5α reduction was obtained by combining the production of 5α -dihydrotestosterone with that of 5α -androstanediol.

DNA estimation.—Tumour DNA content was determined by a modification of the method of Burton (1956).

RESULTS

Tumour growth

The effect of endocrine manipulation was not studied in the primary DMBA-induced tumour or at its initial transplantation. Pattern of growth following oophorectomy and subsequent oestrogen administration is, however, shown in the

Figure for tumours at their 2nd, 3rd, 4th and 7th passages. Tumours at their 2nd passage (TG 5/2) regressed after oophorectomy, but regrew on administration of oestradiol. Oophorectomy of animals bearing TG 5/3 tumours produced only transient tumour regression, and the size 10 days after oophorectomy exceeded that before ablation. In contrast the growth of TG 5/4 tumours appeared not to be affected by oophorectomy though there was evidence for accelerated growth once oestrogen was administered. All subsequent generations of transplanted tumours responded in this way to endocrine manipulation.

Tumour histology

No obvious change in tumour histology was seen during successive transplantation, and fibrosarcomatous development which can appear during transplantation (Horn *et al.*, 1976) was not evident.

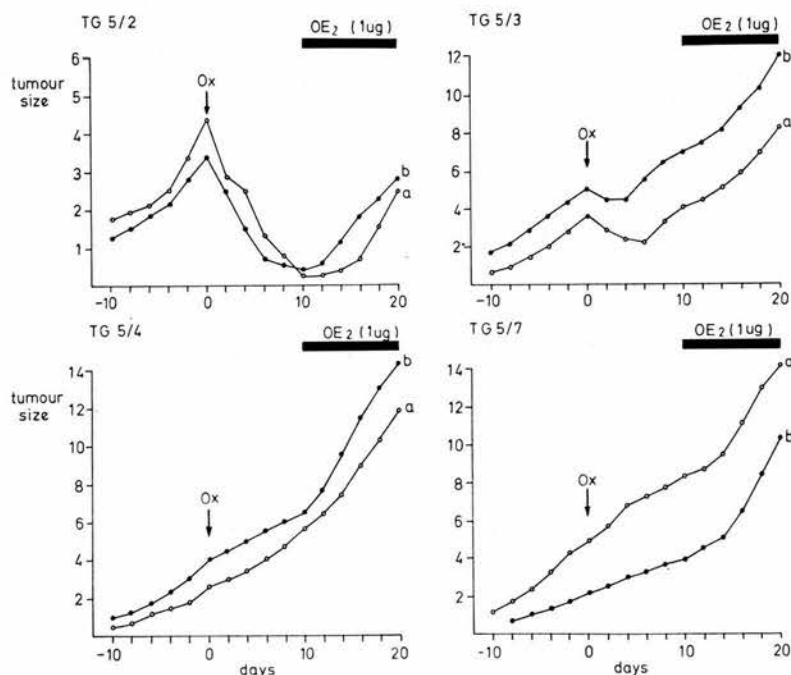


FIG.—Growth patterns of TG 5 transplanted tumours following oophorectomy (Ox) and administration of oestrogen (OE₂). Day 0 represents day of oophorectomy, TG 5/n tumour generation where n is number of passages and (a) and (b) represent individual tumours listed in Table II.

TABLE I.—*Metabolism of [$7\alpha^3H$] testosterone by TG 5 transplanted tumours from endocrine unmanipulated animals*

Transplant generation	DNA content (mg/g tumour)	% Testosterone metabolized	% 5α DHT produced	% 5α Androstenediol produced	% 5α reduction
TG 5/1	5.89	38.30	10.54	23.07	33.61
TG 5/2	6.08	41.62	7.34	23.47	31.81
	6.40	31.68	7.24	20.83	28.07
TG 5/3	7.00	59.05	9.14	40.07	49.21
	5.23	51.96	7.65	36.67	44.32
TG 5/4	7.26	68.62	10.97	52.09	63.06
	5.52	74.18	9.87	59.94	68.81
TG 5/7	5.17	71.89	17.70	51.41	69.11
	4.46	74.25	14.46	55.20	69.66

TABLE II.—*Metabolism of [$7\alpha^3H$] testosterone by TG 5 transplanted tumours from endocrine-ablated, oestrogen-treated animals*

Transplant generation	DNA content (mg/g tumour)	% Testosterone metabolized	% 5α DHT produced	% 5α Androstenediol produced	% 5α reduction
TG 5/2 (a)	5.03	49.89	12.74	30.27	42.81
(b)	4.65	43.25	9.04	30.60	39.64
TG 5/3 (a)	4.26	53.40	12.43	34.58	47.01
(b)	4.28	57.62	12.79	38.15	50.94
TG 5/4 (a)	4.30	69.71	9.54	50.50	60.04
(b)	4.30	69.80	15.21	41.72	56.93
TG 5/7 (a)	5.19	66.90	7.12	48.64	55.76
(b)	4.35	70.57	12.90	50.55	60.45

Tumour steroid metabolism

The results from incubations of tumours with $7\alpha^3H$ testosterone are presented in Tables I and II. In all tumours, 5α reduction of testosterone to 5α dihydrotestosterone and 5α androstenediol accounted for most of the metabolism. In tumours from endocrine-unmanipulated animals (Table I) transformation of testosterone was roughly similar in TG 5/1 and TG 5/2 tumours. However, metabolism was higher in tumours from the TG 5/3 generation, and a further raised level of metabolism was noted in TG 5/4 and TG 5/7 tumours. This change in metabolism with successive transplantation was accounted for by a parallel increase in 5α reduction of testosterone, this being particularly evident in the conversion to 5α androstenediol. Results in tumours from animals after endocrine procedures are shown in Table II. A similar but less marked trend of increasing metabolism and 5α reduction of testosterone with successive generations of

tumours was evident. No progressive changes in tumour DNA content were observed with serial passage in either endocrine-manipulated or unmanipulated animals.

DISCUSSION

The TG5 rat mammary tumour line was derived from a tumour induced by DMBA in a female Sprague-Dawley rat. No information is available on the hormone dependence of this primary tumour or its 1st-generation transplant, but at 2nd passage the growth of the tumour appeared hormone-dependent, regressing after oophorectomy. With successive transplantation, however, the tumour first showed only transient regression (TG5/3) and then no regression after oophorectomy (TG 5/4 onwards). Similar changes after transplantation in tumour growth pattern from ovary-dependent to ovary-independent have been reported in other transplantable rat mammary tumours.

ours (De Sombre *et al.*, 1976; Horn *et al.*, 1976). Whilst later generations of transplantable tumours do not regress after oophorectomy, they may retain some degree of sensitivity to hormones (Hilf, 1972). In the present study the growth of TG 5/4 and TG 5/7 tumours, though not influenced by ovarian ablation, appears to be stimulated by administration of oestrogen to oophorectomized animals. Although oestrogen-receptor activity was detectable in cytosols from TG 5/7 tumours (mean receptor level $3.4 \text{ fmol/mg protein}$, $K_d 0.45 \times 10^{-10} \text{ M}$) the levels were much lower than in DMBA-induced hormone-dependent rat mammary tumours (Hawkins *et al.*, 1978).

In addition to determining the effects of transplantation on tumour endocrine response, successive generations of tumours have been examined for their ability to metabolize testosterone *in vitro*. In all generations of tumours studied, 5α -reduced products were the major metabolites of testosterone. However, with successive transplantation there was an increase in tumour metabolism and 5α reduction of testosterone, such that there was a doubling of 5α reduction between TG 5/1 and TG 5/7 tumours taken from endocrine-unmanipulated animals.

5α androstenediol ($3\alpha 17\beta$) appeared to be the metabolite most consistently affected and it may be that the effects of 5α reduction are secondary to those on 3α hydroxysteroid dehydrogenase. However, under the incubation conditions used, 5α androstenediol was the single greatest 5α reduced product identified, and effects on 5α reduction are most likely to be evident in this metabolite. A similar but smaller rise in 5α reduction with increasing number of passages was evident in endocrine-treated animals. Endocrine manipulation itself has been shown to influence 5α reduction in these tumours: oophorectomy increases the activity and oestrogen administration to oophorectomized animals decreases the 5α reduction (Miller *et al.*, 1979). The effects of oestrogen administration can,

however, be variable, and this may have masked the full effects of transplantation.

It is tempting to speculate that the changes in testosterone metabolism are linked with the transition of tumour hormone dependency. Metabolism in TG 5/2 tumours, which regress after oophorectomy, and TG 5/1 tumours is similar and much lower than in TG 5/4 and TG 5/7 tumours, which do not regress after oophorectomy. Tumours of the TG 5/3 generation, which only show transient regression after oophorectomy, have intermediate metabolism. It is interesting therefore that in the mouse 5α reduction of testosterone is lower in androgen-dependent mammary tumours than in these which are independent (Yamaguchi *et al.*, 1974). Although no significant difference in steroid metabolism was detected between hormone-dependent and independent DMBA-induced rat mammary tumours (King *et al.*, 1965) endocrine-treated animals were used and in the present study effects in such animals were more difficult to detect than in unmanipulated animals. Furthermore the levels of tumour 5α reduction were at least 10-fold less (King *et al.*, 1964) than those reported in the system used in the present study. It is possible that the apparent relationship detected in this study between hormone dependence and steroid metabolism is only causal and that some other feature of transplantation has caused the observed increase in testosterone metabolism.

Difference in tumour growth rate is unlikely, as all tumours were actively growing at the time of study. A simple increase in tumour cellularity is also unlikely, because there was no evidence for an increase in tumour DNA content or histology with transplantation. However, a general increase in cellular metabolism cannot be excluded, and no information is available on the metabolism of other steroid precursors.

Until the specificity of the changes in steroid metabolism is further defined, the physiological relevance of these effects

must remain in doubt. However, to the author's knowledge this is the first documented observation that successive transplantation may affect tumour steroid metabolism. That a change in tumour hormone dependence occurs concurrently and the steroid conversion involved is one which may be hormonally influenced (Miller, 1976a,b,c; Buchan *et al.*, 1976) gives added interest to the findings.

The author is grateful to Professor A. P. M. Forrest for his interest and encouragement and the Cancer Research Campaign for supporting this work with Grant No. SP 1256. The help of Mrs D. Gray, who transplanted and measured the tumours, and Dr A. A. Shivas, who reviewed the tumour histology, is also acknowledged.

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Short Communication**HORMONAL STATUS AND STEROID METABOLISM IN TWO TRANSPLANTABLE RAT MAMMARY TUMOURS**

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HUMAN and rat mammary tumours metabolize steroid hormones and thus have the capacity to modify their local hormonal environment (Adams & Wong, 1968; Jones *et al.*, 1970; King *et al.*, 1964; Miller *et al.*, 1974). In previous studies, we have investigated the metabolism of testosterone by a predominantly ovary-dependent DMBA-induced mammary tumour, and have shown it to be sensitive to levels of other hormones present *in vitro* (Miller, 1976a), or *in vivo* (Miller, 1976b, c; Buchan *et al.*, 1976). We now report a study of the metabolism of androgens by ovary-independent tumours derived by transplantation. By using multiple tumours transplanted to a single rat, it has been possible to examine tumours derived from the same host animal before and after 2 endocrine manipulations.

All animals were of an inbred strain of Sprague-Dawley rat, obtained from the Animal Diseases Research Association (ADRA), Moredun Institute, Edinburgh. Two lines of transplantable tumours were used: TG3 and TG5. These were histologically carcinomas, originally induced in female "ADRA" rats at 50 days of age by i.v. administration of 5 mg of DMBA. Both tumours were then serially transplanted by dorsal skin implantation of tumour fragments into neonatally-thymectomized hosts. At the time of study TG3 was at its 6th passage and TG5 was at its 7th passage.

Three separate portions of a single donor tumour were implanted at different dorsal

sites in each neonatally thymectomized host animal at 50 days of age. Tumours were measured twice weekly throughout the period of study. When the largest tumour reached 1.5×1.5 cm in size it was removed through a dorsal skin incision and the animal was immediately bilaterally oophorectomized. Ten days later, a further tumour was excised. The animals were then given daily injections of oestradiol- 17β ($1 \mu\text{g}$ in 0.2 ml corn oil) for a further 10 days.

The remaining tumour was excised and the animals were killed, no injection of oestradiol being given on the day of death. Eight rats were so treated (4 with TG3 tumours and 4 with TG5 tumours). Control animals (2 with TG3 tumours and 2 with TG5 tumours) were treated similarly except that a sham oophorectomy was performed in place of an oophorectomy and the injection vehicle replaced oestradiol.

After excision, portions of each tumour (0.5 g) were used for steroid-metabolism studies. Each was finally sliced at 0°C in Krebs-Ringer phosphate buffer pH 7.4 (5 ml). An NADPH-generating system and radioactive precursor ($20 \mu\text{Ci}$ of either $7\alpha(^3\text{H})$ dehydroepiandrosterone (DHA) or $7\alpha(^3\text{H})$ testosterone) was added. The incubation systems were shaken at 37°C in an atmosphere of O_2 for 2 h (TG3 series) or 1 h (TG5 series—on account of the high activity found in the tumour lines). The reaction was stopped by adding methanol to 80% v/v and the incubations stored at

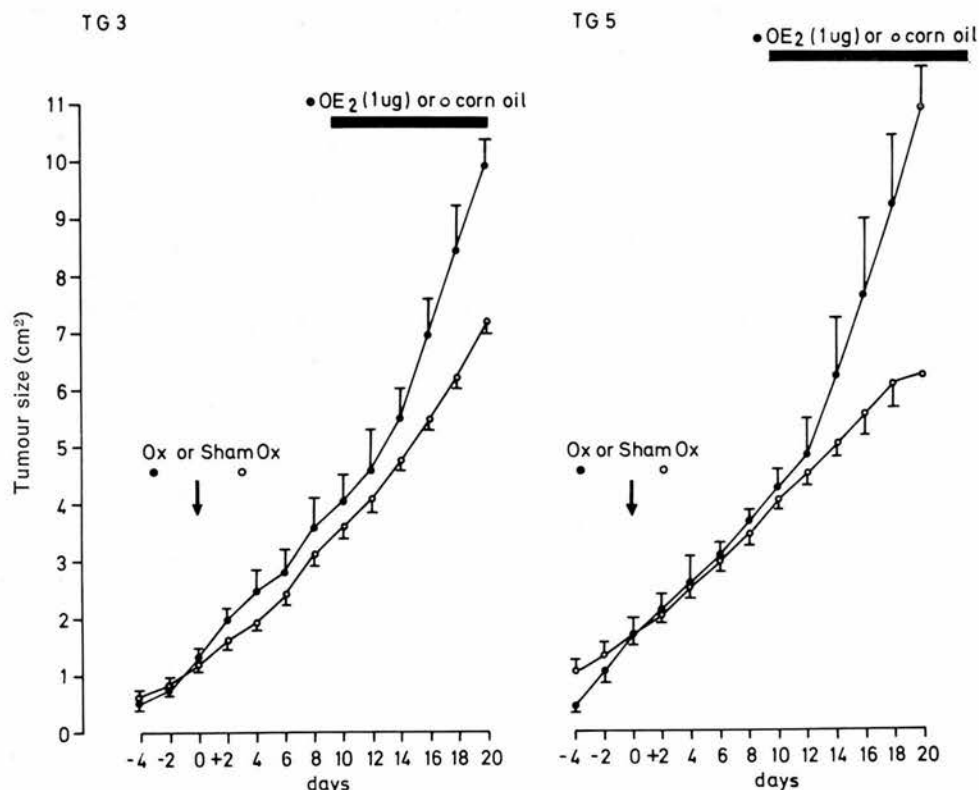


FIGURE.—Growth patterns of TG3 and TG5 transplantable tumours. Solid dots are values for experimental animals with oophorectomy followed by oestrogen (OE_2). Each point represents mean size of the 4 tumours (each from a separate animal) which were subject to both hormone manipulations, with the exception of the final point for TG5 (mean of 3 tumours: one animal killed on Day 18). Open dots are values for control animals with sham oophorectomy followed by corn oil. Each point represents the mean of 2 tumours (each from different animals) which were subject to both manipulations, with exception of the final point for TG5 (value for a single tumour: one animal killed on Day 18). Bars represent s.e. mean. Day 0 represents day of oophorectomy or sham operation.

—10°C until the metabolites were characterized. To measure losses, 500 µg of each non-radioactive carrier steroid to be investigated was added. The metabolites were then extracted and separated into individual metabolites as described previously (Miller *et al.*, 1974). Purification of all steroid fractions except 5α androstenediol was achieved by sequential acetylation and hydrolysis; 5α androstenediol by sequential oxidation and reduction. Methods for derivative formation and characterization of metabolites have been described previously (Miller *et al.*, 1974). Metabolism and conversion of precursors were measured by determining

the incorporation of radioactive label into the appropriate metabolites after correction for losses. 5α reduction of testosterone was estimated by combining the production of 5α dihydrotestosterone with that of 5α androstenediol.

The pattern of tumour growth throughout the endocrine procedures investigated is shown in the Figure. Tumour growth was continuous after oophorectomy, but after the administration of oestradiol there was some evidence for accelerated tumour growth, especially in the TG5 line. Sham oophorectomy and administration of corn-oil vehicle had little effect on tumour growth.

TABLE I.—*Effects of endocrine status on metabolism of $7\alpha(^3H)$ testosterone by TG3 transplanted tumours*

Group	No. of rats	Endocrine status of tumour donors	% Testosterone precursor	
			Unmetabolized	Metabolized by 5α reduction
Control	2	Intact	2.6, 4.5	65.2, 61.0
		Sham oophorectomized 10 days earlier	2.0, 3.1	66.3, 64.6
		Sham oophorectomized 20 days earlier, +corn oil for last 10 days	2.0, 3.8	72.5, 62.7
Experimental	4	Intact	6.5, 4.3, 3.7, 7.8	57.5, 56.2, 59.5, 75.4
		Oophorectomized 10 days earlier	2.0, 0.9, 1.5, 0.7	99.3, 86.2, 98.1, 96.1
		Oophorectomized 20 days earlier	13.9, 9.3, 5.2, 7.2	82.4, 68.4, 86.5, 77.2
		+1 μ g oestrogen in corn oil for last 10 days		

TABLE II.—*Effects of endocrine status on metabolism of $7\alpha(^3H)$ testosterone by TG5 transplanted tumours*

Group	No. of rats	Endocrine status of tumour donors*	% Testosterone precursor	
			Unmetabolized	Metabolized by 5α reduction
Control	2	Intact	17.8, 32.0	82.4, 66.1
		Sham oophorectomized	17.0, 21.0	83.7, 75.1
		Sham oophorectomized +corn oil	14.9, —	80.5, —
Experimental	4	Intact	25.8, 21.2, 27.4, 26.7	69.7, 74.8, 68.9, 67.0
		Oophorectomized	13.8, 15.1, 16.3, 16.2	81.6, 82.1, 86.8, 82.3
		Oophorectomized +oestrogen	33.1, 19.4, 25.2, —	55.8, 68.5, 70.0, —

* See Table I for details.

The general histological appearance of both tumour lines was not changed by either oophorectomy or oestrogen administration, in terms of cell number, cell type, necrosis or polyploidy.

The % metabolism of testosterone and its conversion to 5α reduced products by TG3 tumours are shown in Table I. All TG3 tumours, irrespective of the animal from which they were derived or its endocrine status, metabolized large amounts (86–99%) of the testosterone precursor. Despite this high metabolic activity, oophorectomy caused an increase in tumour testosterone metabolism in each animal. This effect was consistently reversed by the administration of oestrogen to the oophorectomized animals. In each animal, oophorectomy was also associated with an increase in tumour 5α reduction of

testosterone. Subsequent oestrogen administration to oophorectomized animals then led to decreased tumour production of 5α reduced metabolites, although tumour 5α reduction still remained higher than that in corresponding tumours from the intact individuals. Of the individual 5α reduced metabolites investigated, the effects were most marked on 5α androstanediol, perhaps because its production was at least twice that of 5α dihydrotestosterone in all TG3 tumours. The rise in 5α reduced metabolites produced by oophorectomy was significantly different from the small change caused by sham ablation in the control group ($P < 0.05$ as based on t test of the logarithms of the ratio between the groups.) Similarly, the fall in tumour 5α reduction apparent after administration of oestradiol was significantly different

TABLE III.—*Effects of endocrine status on metabolism of $7\alpha(^3\text{H})$ DHA by TG3 transplanted tumours*

Group	No. of rats	Endocrine status of tumour donors*	% DHA precursor	
			Unmetabolized	As $\Delta 5$ androstenediol
Control	2	Intact	64.8, 76.8	15.8, 19.7
		Sham oophorectomized	59.8, 71.9	17.8, 21.9
		Sham oophorectomized + corn oil	60.1, 70.3	19.2, 21.1
Experimental	4	Intact	75.3, 74.6, 64.9, 81.3	15.0, 17.9, 18.4, 13.6
		Oophorectomized	74.5, 70.8, 57.7, 84.8	15.5, 21.0, 22.3, 18.0
		Oophorectomized + oestrogen	71.3, 69.2, 76.5, 66.7	13.9, 22.3, 17.3, 23.3

* See Table 1 for details.

from the effect of injection vehicle in control animals ($P < 0.01$).

The results obtained from incubations of TG5 tumours are presented in Table II. As with the TG3 series, oophorectomy was associated in each animal with an increase in tumour metabolism and 5α reduction of testosterone; these effects were reversed after administration of oestradiol to the animals. Both 5α dihydrotestosterone and 5α androstenediol were equally affected by treatment. Transformation to both these 5α reduced metabolites was alone sufficient to account for the changes in testosterone metabolism produced by hormonal manipulation.

The metabolism of $7\alpha[^3\text{H}]\text{DHA}$ was investigated in duplicate portions of the same TG3 tumours used to study the metabolism of testosterone. The distribution of radioactivity from $7\alpha[^3\text{H}]\text{DHA}$ after incubation is shown in Table III. The metabolism of this steroid (16–43%) was less extensive than that of testosterone. Oophorectomy and oestrogen treatment failed to influence the overall metabolism of DHA or the production of its major metabolite, $\Delta 5$ androstenediol. Metabolism of $7\alpha[^3\text{H}]\text{DHA}$ was also similar in tumours from control animals subjected to sham oophorectomy followed by injection of vehicle.

As both TG3 and TG5 tumours maintain their histological appearance during serial and multiple transplantations, they provide useful models for studying the effects of sequential operations within individual animals, without the need to take

biopsy samples from individual tumours, a procedure which alone may change tumour behaviour. In the present communication we have utilized these tumour lines to study the effects of endocrine manipulation on steroid metabolism.

Compared with ovary-dependent DMBA-induced tumours (Miller, 1976b), both the ovary-independent transplantable tumour lines had a high capacity to metabolize ^3H -testosterone, especially by 5α reduction. In both TG3 and TG5 tumours, oophorectomy was associated with increased metabolism of testosterone, whereas administration of oestrogen reversed the effect. These changes could be accounted for largely by parallel changes in the production of 5α reduced metabolites. However, it is unlikely that the changes were nonspecific since the overall metabolism of dehydroepiandrosterone and conversion to its major metabolite $\Delta 5$ androstenediol were, by contrast, uninfluenced by endocrine manipulation. Furthermore, there were no obvious histological changes in tumour appearance, such as increase in cell number after oophorectomy or cell death after oestrogen treatment, which might directly account for the changes in testosterone metabolism resulting from endocrine manipulation.

The pattern of changes in testosterone metabolism after endocrine manipulation does not differ from that observed previously with DMBA-induced ovary-dependent tumours (Miller, 1976b). Thus the sensitivity of steroid metabolism to endocrine manipulation does not differenti-

ate between tumours *dependent* on the ovary for their growth and those which are not. However, the transplantable tumours were derived originally from DMBA-induced tumours, and at least one of the lines (TG5) was ovary-dependent in its first and second transplant generations. Since (1) oestrogen receptors are present in the tumours (Hawkins *et al.*, 1978) and (2) tumour growth, whilst not ovary-dependent, may be enhanced by oestradiol-17 β administration (Figure), it seems likely that these tumours have retained some hormonal *sensitivity* during transplantation.

Similar progressive changes in hormone dependency with succeeding transplant generations have been shown for other transplantable tumours (Sluyser & Van Nie, 1974; DeSombre *et al.*, 1976; Horn *et al.*, 1976). The transplantable TG3 and TG5 tumours may thus resemble the transplantable R3230AC tumour, which has been described by Hilf (1972) as ovary-independent but hormone-sensitive.

It is thus possible that the changes in steroid metabolism observed in these transplantable ovary-independent TG3 and TG5 tumours following endocrine manipulation are related, at least in part, to some residual hormonal sensitivity.

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We are also indebted to Mr D. Drewitt and Mr I. W. J. Wallace who originally induced and transplanted the tumour lines used in this study, Mrs D. Gray who subsequently transplanted and measured later generations of the tumours, and Dr A. A. Shivas who reviewed the histology of the tumours.

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Short Communication

STEROID METABOLISM BY HUMAN NORMAL THYROID, NODULAR GOITRE AND THYROID CANCER

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PREVIOUS studies (Adams and Wong, 1968; Jones *et al.*, 1970; Miller *et al.*, 1973) have shown that both normal and neoplastic breast tissues perform steroid conversions similar to those in the classic steroid secreting organs of the ovary, testis and adrenal. The present paper describes a study of human thyroid tissue used to determine the potential of normal and neoplastic endocrine tissue of another type to metabolize C19 steroids *in vitro*.

In particular, attention was focused upon the transformation of DHA to testosterone and the 5α reduction of testosterone, activities which produce metabolites of greater biological activity than the parent substrate.

MATERIALS AND METHODS

Three specimens of thyroid cancer of adenocarcinomatous (or follicular) type (Fig. 1) and 3 of simple nodular goitre (Fig. 2) were examined. Normal thyroid tissue also was obtained from 3 patients; one of these had no thyroid disease, one had a gland containing a carcinoma, the other a nodular goitre. In the latter two instances the tissue was histologically normal. All material was freshly obtained from the operating theatre and taken in the frozen section laboratory for immediate incubation.

All tissues were processed at 0°C until incubation was carried out (within 30 min of tissue removal). The specimens were finely sliced and incubated for 2 h at 37°C in Krebs-Ringer phosphate buffer, pH 7.4 (10 ml/g tissue) containing an NADPH

generating system (200 μ mol glucose-6-phosphate, 25 mg NADP and 50 units glucose-6-phosphate dehydrogenase) and the radioactive precursors: 20 μ Ci(7α - 3 H)dehydroepiandrosterone (DHA) and 2 μ Ci(4 - 14 C) testosterone.

The steroid interconversions were then determined by measuring the percentage incorporation of the appropriate radioactive labels into the various purified metabolites. The methods for steroid purification and characterization have already been described in detail (Miller, Hamilton and Forrest, 1974).

RESULTS

The results of these experiments are presented in the accompanying Table. Normal, goitrous and malignant thyroid tissue were all capable of extensively metabolizing the DHA precursor. Testosterone was isolated as a metabolite of DHA in each type of thyroid tissue, but it accounted for only a small amount of the precursor used.

By contrast, the metabolism of testosterone by the same tissues was slight and in 2 of the 3 normal thyroid tissues, metabolism was entirely absent. In comparison with normal thyroid, the metabolism of testosterone was greater in nodular and malignant tissue. Both 5α dihydrotestosterone and 5α androstanediol were characterized as metabolites of testosterone in nodular and malignant tissue, but there was no evidence for 5α reduced products

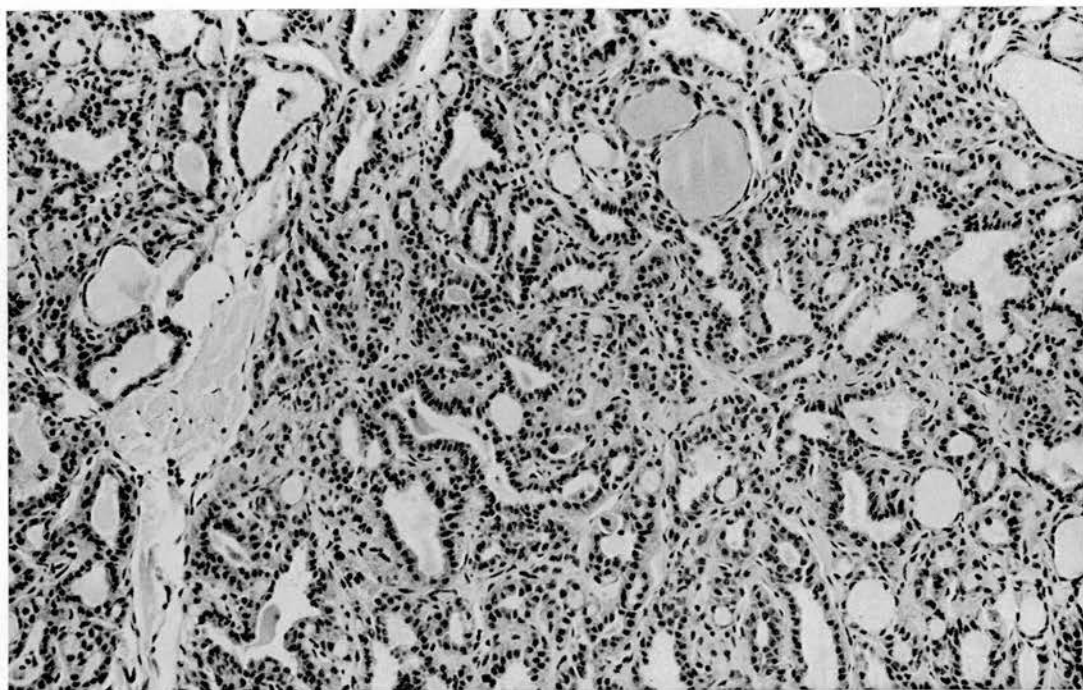


FIG. 1.

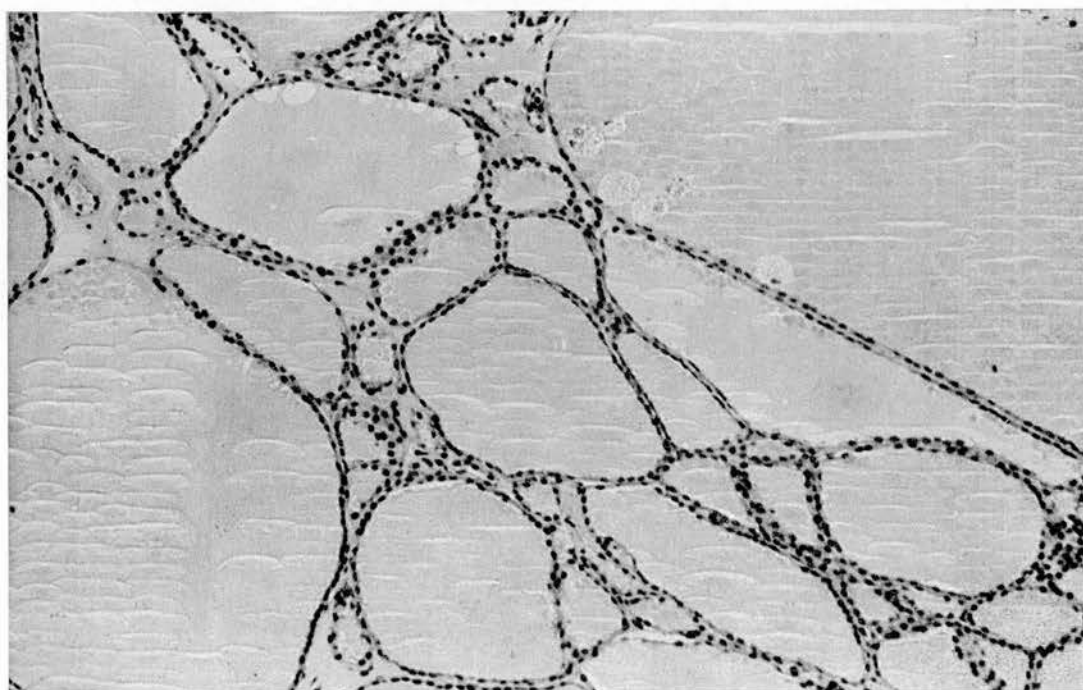


FIG. 2.

TABLE.—*Metabolism of (7 α -³H)dehydroepiandrosterone and (4-¹⁴C)testosterone by Human Thyroid Tissue*

Patient	Age and sex	Histology of tissue	DHA		Testosterone		
			% metabolized	% as testosterone	% metabolized	% as dihydro-testosterone	% as androstenediol
1	8M	Adenocarcinoma	38.40	0.17	18.80	0.24	0.18
		Normal tissue	52.50	0	0	0	0
2	27M	Adenocarcinoma	92.91	3.16	10.10	0.14	—
3	67F	Adenocarcinoma	95.17	0	10.10	0.39	0.49
4	58F	Nodular goitre	79.10	0.63	4.99	0.61	0.15
		Normal tissue	51.13	0.53	0	0	0
5	25F	Nodular goitre	59.37	1.38	16.80	—	—
6	54F	Nodular goitre	65.67	0.26	2.61	0.48	0.13
7	18F	Normal tissue	65.70	0.21	2.80	0	0

in normal tissue even when it was associated with neoplastic tissue possessing 5 α reductase activity.

Evidence for the conversion of either DHA or testosterone to 16 α hydroxylated derivatives or oestrogen was not detected in any incubation material.

DISCUSSION

These results confirm the observations of Schneider *et al.* (1972) that thyroid tissue can metabolize DHA extensively. This ability is not confined to normal tissue, but is also present in both goitrous and malignant states.

High metabolism was restricted to DHA and the same thyroid tissues metabolized much less of the testosterone precursor. Small but significant amounts of both 5 α dihydrotestosterone and 5 α androstenediol were formed from testosterone in incubations of both goitrous and malignant thyroid. No such evidence for the 5 α reduction of testosterone was found in the 3 incubations of normal thyroid, despite the fact that thyroxine stimulates 5 α reduction in other tissues (Tomkins, Gordon and McGuire, 1960).

In two instances, 5 α reduction in pathological thyroid tissues was obtained even when the adjacent normal thyroid did not display the activity. Although the number of incubations is too small to draw firm conclusions, the possibility exists that pathological change in the

thyroid is accompanied by the acquisition of potential to perform steroid conversions not shown in normal tissue.

In a previous report (Miller *et al.*, 1973) we have described the metabolism of DHA and testosterone by human normal and neoplastic breast tissues, using identical methods to those used for the thyroid tissue studied in this communication. It is worth noting that the amounts of the individual steroid precursors metabolized were different in the two types of tissue, thyroid showing greater metabolism of DHA but less of testosterone, compared with breast tissue.

Nevertheless, with the exception of normal thyroid tissue, the production of 5 α dihydrotestosterone and 5 α androstenediol from testosterone was quantitatively similar in thyroid and breast tissues, as was the conversion of DHA to testosterone in all tissues examined. This suggests that these conversions are relatively non-specific. Further evidence for this is the demonstration of similar transformations in human skin (Hodgins, 1971; Wilson, 1972) and metastatic deposits of a bronchiogenic cancer (Miller, unpublished results).

We are grateful to Professor A. R. Currie for allowing us to use fresh material from the frozen section laboratory.

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Steroid interconversions by metastatic deposits of a human bronchogenic carcinoma

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Minces of a cervical node involved by metastases of a bronchogenic carcinoma metabolized extensively both C₂₁ and C₁₉ steroid precursors. Pregnenolone and dehydroepiandrosterone (DHA) were transformed largely to Δ -5-androstenediol. Both 5- α -dihydrotestosterone and 5- α -androstenediol were metabolites of testosterone.

Introduction

Bronchogenic carcinomas are known to be associated with a variety of paraendocrine syndromes (Bower & Gordon, 1965), these being due to elaboration of protein and polypeptides with hormone-like activity (Mason, Ratcliffe, Buckle & Mason, 1972). No information is available of the steroidogenic capacity of bronchogenic cancer and reports of steroid effects by them are rare (Daughtrey, Chesney, Spear, Gentsh & Larsen, 1967). In this report we describe a study of steroid biosynthetic activity in a secondary deposit of bronchogenic cancer in a lymph node.

Methods

The subject was a 74-year old man who first presented with enlarged glands in the neck. On biopsy these were shown to be involved by metastatic deposits from an anaplastic bronchogenic tumour. One of the nodes was taken for incubation and a typical microscopic field of the tissue is shown in Figure 1(a) and (b).

Incubation conditions

The tissue was finely sliced and split into 3 equal portions. Krebs Ringer phosphate buffer pH 7.4 (10 ml/g tissue), an NADPH generating system and 50 μ Ci of either [7- α -³H]pregnenolone, [7- α -³H]DHA, or [7- α -³H]testosterone were added. Each was incubated for 2 h at 37°C in an atmosphere of oxygen. The steroid metabolites were extracted, purified and characterized as described elsewhere (Miller, Hamilton & Forrest, 1974). Conversions were determined by measuring the percentage incorporation of ³H into the metabolites after correction for manipulative losses.

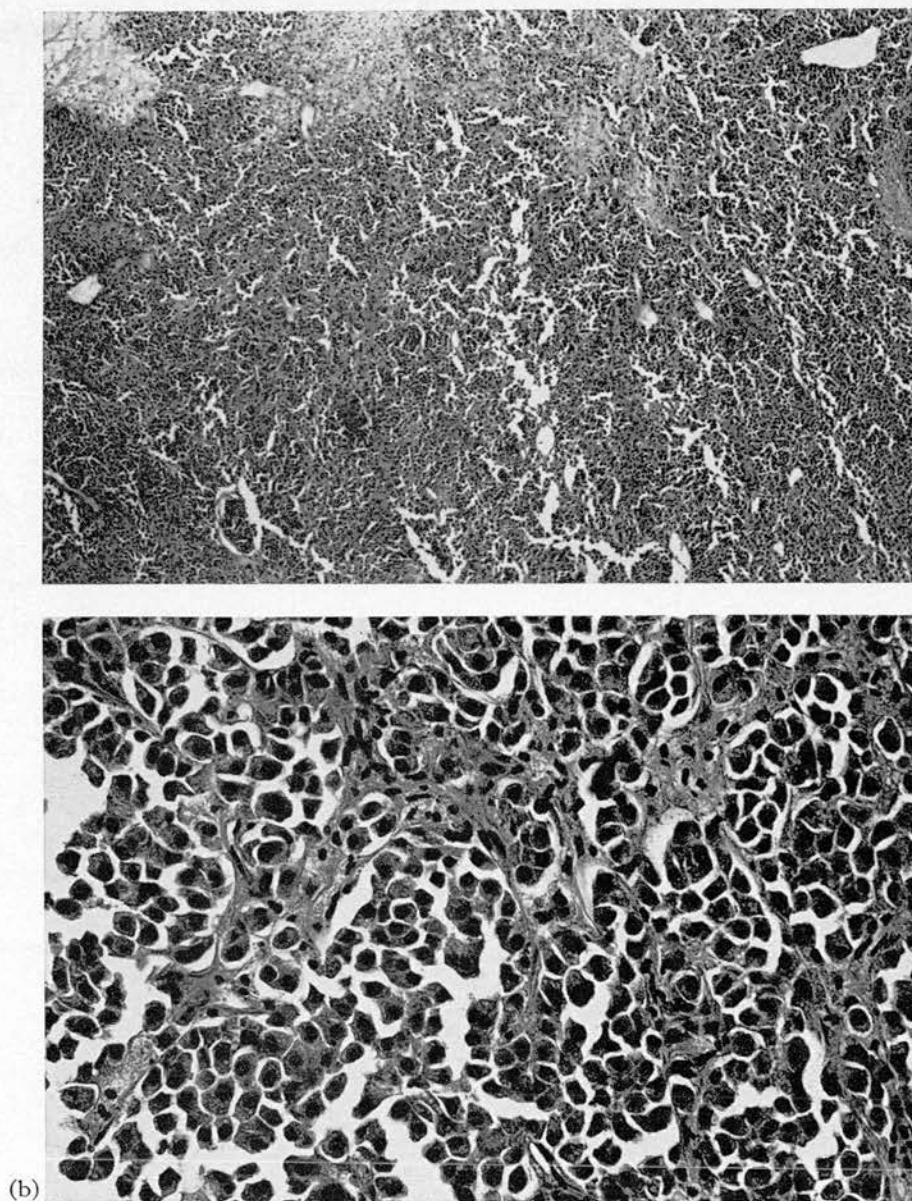


Figure 1. (a) Low power view of the tumour—anaplastic carcinoma showing some local necrosis (right upper field). H+E $\times 100$; (b) Detail of (a)—moderate cellular pleomorphism is present and a large cell type predominates. The morphology is typical of many bronchogenic carcinomas. H+E $\times 450$.

Results

The results are presented in Table 1 and summarized in Figure 2. All 3 substrates were extensively metabolized. The presence of desmolase activity was demonstrated by the conversion of pregnenolone to Δ -5-androstenediol and DHA. Only a very small transformation of pregnenolone to progesterone was obtained. The major metabolite of DHA was also Δ -5-androstenediol, but small amounts of Δ 4-androstenedione and testosterone were also produced. Testosterone itself was metabolized

to both 5- α -dihydrotestosterone and 5- α -androstenediol, but a large proportion of the substrate remained unaccounted for. There was no evidence for the production of oestrogen from any of the precursors.

TABLE 1. % Metabolism of 7- α - 3 H steroid precursors by bronchogenic carcinoma

	Pregnenolone	DHA	Testosterone
Pregnenolone	60.82	—	—
Progesterone	0.08	—	—
DHA	0.31	55.76	—
Δ -5-Androstenediol	14.18	37.25	—
Testosterone	—	0.06	36.40
Δ -4-Androstenedione	—	0.05	—
5- α -Dihydrotestosterone	—	—	0.43
5- α -Androstenediol	—	—	12.10

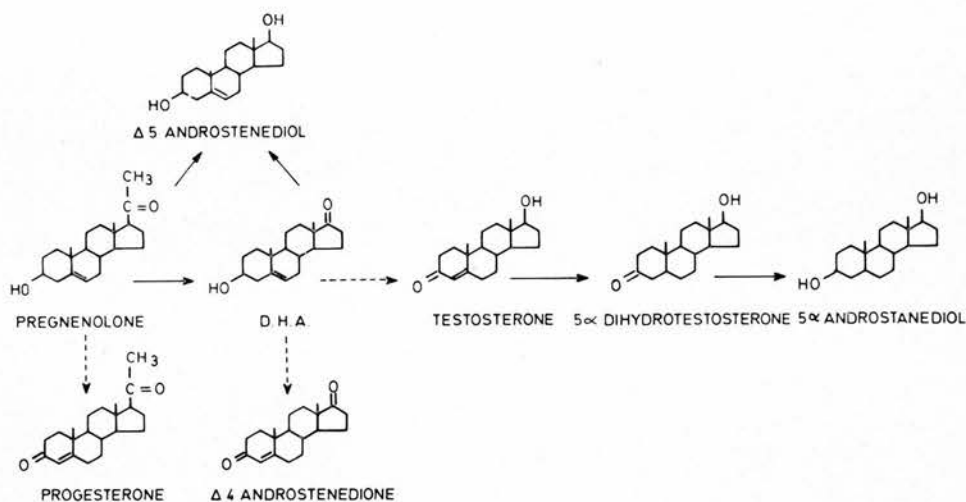


Figure 2. Pattern of steroid metabolism present in tumour studied.

Discussion

These results indicate that bronchogenic carcinoma may have the potential to synthesize steroid hormones.

Precursors such as pregnenolone and DHA, which are normally present in the plasma in high concentrations, were extensively metabolized. The production from pregnenolone of high amounts of Δ -5-androstenediol and only small amounts of progesterone would seem to indicate relatively high desmolase and reductive activities in comparison with oxidative pathways. Preference for reductive metabolism was also confirmed by the production from DHA of high amounts of Δ -5-androstenediol, but only low amounts of Δ -4-androstenedione. The production of 5- α -dihydrotestosterone and high amounts of 5- α -androstenediol from testosterone is interesting in view of the high androgenic activity of these compounds in prostate (Bruchovsky & Wilson, 1968) and skin (Wilson & Walker, 1969).

These steroid conversions are qualitatively identical to those which we, and others, have demonstrated in the breast and its tumours (Miller *et al.*, 1973), and in cancer of the thyroid (Miller, Shivas & Forrest, 1974). Quantitatively the metabolism of each precursor was greater for the bronchogenic tumour than that for breast cancer (Miller *et al.*, 1973), or for lymph nodes involved by breast cancer (unpublished results). However, the demonstration that bronchogenic carcinoma can synthesize active androgenic steroids may be of importance in view of the reported incident of masculinization associated with the presence of a bronchial tumour (Daughtrey *et al.*, 1967)—symptoms such as acne, male hair distribution, breast atrophy and clitoral enlargement which disappeared on tumour excision, could be explained on the basis of excessive production of androgenic steroids by the tumour.

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SECTION C

URINARY STEROIDS IN PATIENTS WITH BREAST DISEASE

URINARY AETIOCHOLANOLONE IN PATIENTS WITH EARLY BREAST CANCER FROM SOUTH EAST SCOTLAND AND SOUTH WALES

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Summary.—Urinary aetiocholanolone levels have been measured in 417 women aged between 20 and 70 years. The women were drawn from South East Scotland and South Wales and consisted of patients with either benign or malignant disease of the breast and control patients suffering from no detectable breast disorder. The pattern of aetiocholanolone excretion with respect to age and menopausal status has been defined in each group of patients.

No significant differences in urinary levels have been detected between patients with breast disease, whether benign or malignant, and control patients. More detailed examination of the 201 women with early cancer of the breast has also shown that there is no consistent correlation between pre-operative aetiocholanolone levels and factors of prognostic significance detectable at the time of primary treatment—tumour size, grade, round cell infiltration, histological involvement of nodes by tumour and the clinical palpability of lymph nodes.

It would seem, therefore, that the prognostic value of pre-operative aetiocholanolone measurements is somewhat limited in patients with early breast cancer. It is noted, however, that low levels of aetiocholanolone are associated with post-menopausal patients, a group in which the prognosis is generally poorer than that in pre-menopausal women.

CONSIDERABLE interest in the relationship of urinary androgen metabolites with breast cancer has been provoked by the studies of Bulbrook and his colleagues (Bulbrook *et al.*, 1962). These workers have indicated that (a) some women with advanced cancer have subnormal levels of urinary aetiocholanolone and are unlikely to respond to major endocrine ablation (Atkins *et al.*, 1968*a, b*); (b) a proportion of women with early breast cancer also have abnormally low aetiocholanolone levels (Bulbrook *et al.*, 1962) and that this is associated with poor prognosis (Hayward and Bulbrook, 1968); (c) "normal" women who subsequently develop breast cancer have lower mean excretion of aetiocholanolone than

matched controls (Bulbrook, Hayward and Spicer, 1971).

Whilst it is now accepted that low aetiocholanolone levels are associated with a proportion of patients with advanced breast cancer who have a poor prognosis (Kumaoka *et al.*, 1968; Cameron *et al.*, 1970), the situation in patients with early disease remains equivocal, several studies indicating that aetiocholanolone levels are normal in these women (Alquist, Jackson and Stewart, 1968; Wade *et al.*, 1969; Cameron *et al.*, 1970). With the recent finding that C₁₉ androgenic steroids may act as precursors for tumour growth promoting hormones (Miller and Forrest, 1974), it is even more important to resolve this conflict.

In an effort to do so, we have retrospectively examined the results of urinary aetiocholanolone measurements performed in two major centres in normal women and in patients with either early cancer or benign disease of the breast. These have been analysed with regard to age and menopausal status, in order to define the normal pattern of excretion in patients and controls, and then related to other factors of known prognostic significance.

PATIENTS AND METHODS

Urine was obtained from 417 hospital in-patients in Cardiff and Edinburgh. Patients were excluded from the study if they had known endocrine disease, had been receiving steroid preparations of any type or had a history of cancer at any site other than in the breast.

Control patients.—These were 23 women from Cardiff and 22 from Edinburgh who were admitted to hospital for elective surgery for conditions not involving the breast. None was suspected of having malignant disease of any kind. The majority were to undergo cholecystectomy, but others included patients awaiting surgery for hernias, duodenal ulcers and varicose veins. These patients were therefore subjected to similar pre-operative stress as subjects with breast disorders.

Benign breast disease.—The 163 women in this group had benign disease of the breast proved by excision biopsy. No further histological classification of the 76 women from Cardiff was made but the pathological records were checked to ensure that the diagnosis of benign disease was correct.

Sufficient numbers of pre-menopausal women from Edinburgh with benign breast disease were studied to subdivide this group further.

Group 1—fibrocystic disease of the breast (17 patients) (those patients in whom the predominant histological abnormalities were the presence of fibrosis and cyst formation);

Group 2—epitheliosis (17 patients) (lesions in which epitheliosis of a marked degree was present);

Group 3—fibroadenomata (25 patients).

Cancer patients.—There were 60 patients from Cardiff and 141 patients from Edinburgh with invasive carcinoma of the breast. All of these cases were deemed to have operable or early cancer of the breast and the majority were of international clinical Stages I and II. A few cases were in international clinical Stage III, solely on account of a primary tumour of a size greater than 5 cm diameter.

Menstrual status.—Each of the groups has been further divided according to their menstrual status, by the following criteria:

Pre-menopausal—patients with regular menstrual periods;

Menopausal—patients whose periods were diminishing in frequency or whose last period was within 2 years of primary treatment;

Post-menopausal—patients whose last period had occurred more than 2 years before treatment.

Tumour grade.—Histological grade of the primary tumour was assessed in 191 of the 201 patients, using the criteria of Patey and Scarff (1928, 1929). In this classification, increasing grade reflects an increase in tumour malignancy (Champion and Wallace, 1971).

Round cell infiltration.—At the time of grading, a semi-quantitative assessment of round cell infiltration was also carried out, as described by Champion, Wallace and Prescott (1972).

Tumour size.—The clinical size of the tumour was measured by 2 independent observers at the time of presentation. The tumour was measured in 2 planes at right-angles and the larger diameter was recorded in cm.

Lymph node involvement.—The clinical status of the axillary nodes was assessed by 2 observers before operation. Microscopical examination for the presence of tumour was carried out in all cases from which lymph nodes had been removed. In Cardiff, where the standard operations were either a simple mastectomy with pectoral node biopsy or a modified radical mastectomy, axillary node histology was available. In Edinburgh, where simple and radical mastectomies were performed, it was possible to make a histological assessment of the radical mastectomy group only (68 patients), no axillary nodes being avail-

able from those patients undergoing simple mastectomy.

Urine collection and analysis.—Urine was collected from all patients pre-operatively; in Cardiff for 48 h and in Edinburgh for 24 h before operation.

The techniques used for estimation of aetiocholanolone were based on that described by Thomas and Bulbrook (1964), and Thomas (1965). Different modifications of these basic methods were introduced separately in Cardiff (Cameron *et al.*, 1970) and Edinburgh (Sneddon, 1969). These variations have led to minor differences between centres with respect to extraction, purification and measurement of aetiocholanolone, all of which are unlikely to affect quantitation. One major difference between the methods employed in the 2 centres, however, was the use of conjugated internal standards in Cardiff and free steroid standards in Edinburgh in order to monitor manipulative losses. This is likely to result in different absolute values for aetiocholanolone in any given specimen of urine. The results from the 2 centres are therefore presented separately. Nevertheless, any differences in aetiocholanolone values between the centres ought to be consistent, and the same relationship between aetiocholanolone and other parameters therefore would be expected in results from both Cardiff and Edinburgh.

RESULTS

The mean and standard deviation for the ages of the patient groups under study in Cardiff and Edinburgh is shown in Table I. The pre-menopausal, benign and control groups from Cardiff are considerably younger than the corresponding cancer group. To achieve comparability, only individuals aged 30 or more have been compared in the subsequent tests of significance (see below).

As statistical analysis of urinary steroids with age revealed a log normal distribution (Cameron *et al.*, 1970), aetiocholanolone levels have been expressed in logarithms to the base 10 of the concentration ($\mu\text{g}/24\text{ h}$).

Age and menopausal status

Scattergrams relating aetiocholanolone levels to age and menopausal status in the patients with early breast cancer, from Cardiff are shown in Fig. 1 and from Edinburgh in Fig. 2. These figures also show the regression lines of aetiocholanolone with age for the pre- and post-menopausal patient groups. The regression coefficients for these lines do not differ significantly from zero. Age *per se*, therefore, has little effect on aetiocholanolone excretion in patients from either city. There is, however, a significant difference between the mean levels of aetiocholanolone in the pre- and post-menopausal patients, there being a substantial reduction in aetiocholanolone excretion after the menopause.

Analysis of the control and benign disease groups (restricted to 30 years and older) also revealed a similar pattern. Once subdivided into pre- and post-menopausal patients, age had no significant effect on the level of urinary aetiocholanolone. The pre-menopausal patients, however, showed significantly increased levels compared with post-menopausal patients (Fig. 3, 4).

Information was available from a number of patients under the age of 30 years—15 patients with benign disease and 5 control patients from Cardiff. These data are included in Fig. 5 and 6, which show the relationship between the aetiocholanolone excretion for all of

TABLE I.—Mean Age and Standard Deviation of Patient Groups under Study

	Pre-menopausal		Post-menopausal	
	Cardiff	Edinburgh	Cardiff	Edinburgh
Controls	33.0 \pm 12.3	39.1 \pm 7.3	63.1 \pm 7.2	60.7 \pm 5.0
Benign disease	35.6 \pm 8.7	40.3 \pm 5.4	50.0 \pm 5.2	57.0 \pm 6.3
Cancer	43.1 \pm 6.78	44.0 \pm 4.5	60.7 \pm 5.8	59.7 \pm 6.5

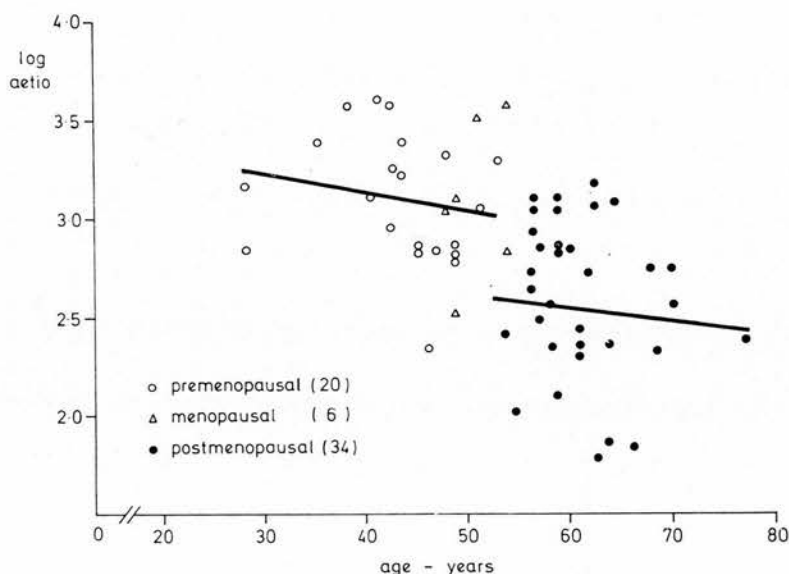


FIG. 1.—Scattergram relating aetiocholanolone levels to age and menopausal status in 60 patients with early breast cancer from Cardiff.

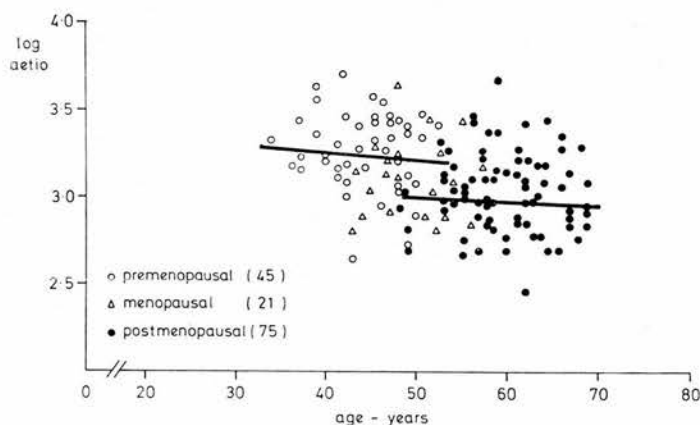


FIG. 2.—Scattergram relating aetiocholanolone levels to age and menopausal status in 141 patients with early breast cancer from Edinburgh.

the pre-menopausal control patients and for those with benign breast disease. In the benign group, there is a gradual increase in aetiocholanolone levels up to the age of 30 years, beyond which the mean level of excretion becomes relatively constant. This relationship is

not apparent in the small number of women in the control group.

Variation with breast disease

This information is presented in Fig. 3 and 4. Analysis of variance showed no significant difference in mean aetio-

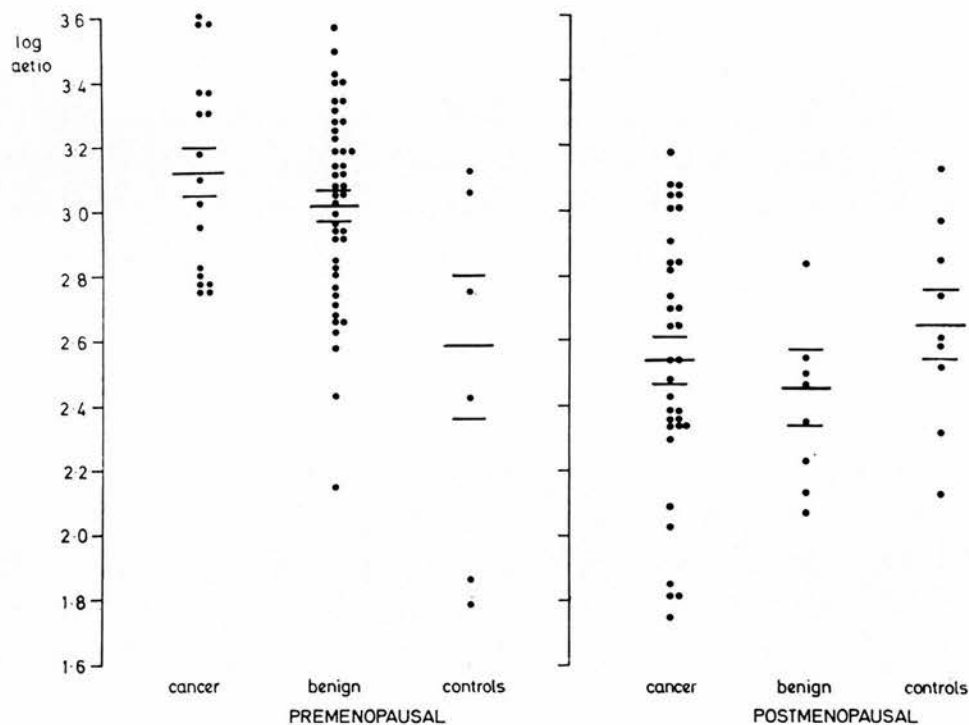


FIG. 3.—Urinary aetiocholanolone levels by menopausal status in control subjects and patients with early breast cancer and benign breast disease from Cardiff (lines refer to mean \pm standard error for the Groups).

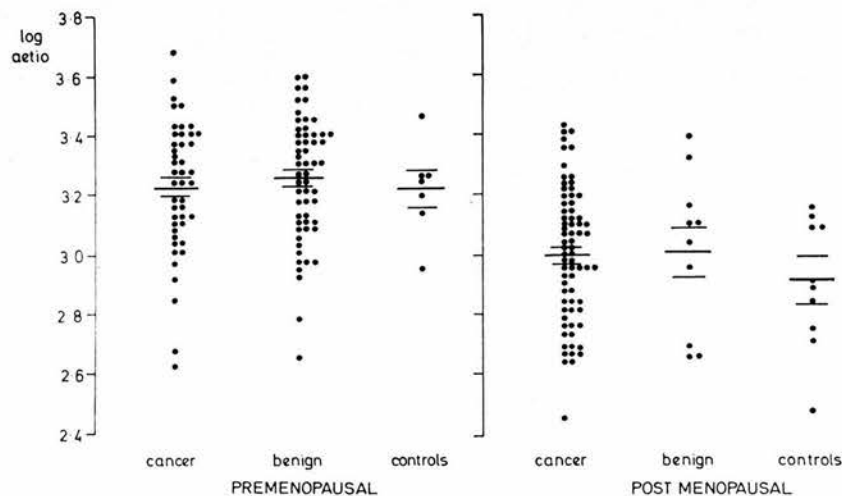


FIG. 4.—Urinary aetiocholanolone levels by menopausal status in control subjects and patients with early breast cancer and benign breast disease from Edinburgh (lines refer mean \pm standard error for the groups).

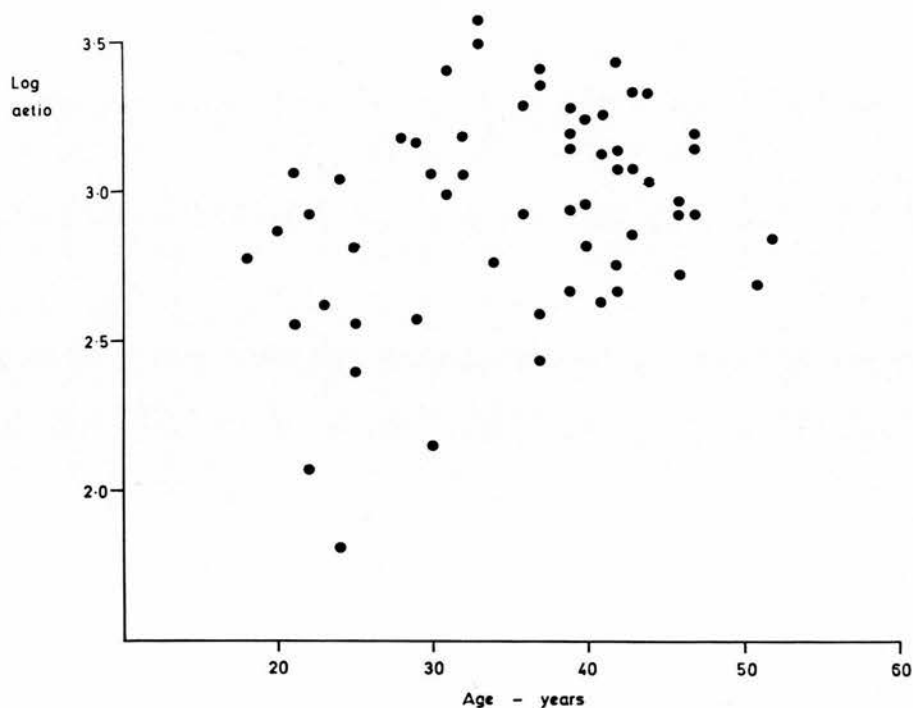


FIG. 5.—Scattergram of aetiocholanolone levels against age in 58 premenopausal patients with benign breast disease from Cardiff.

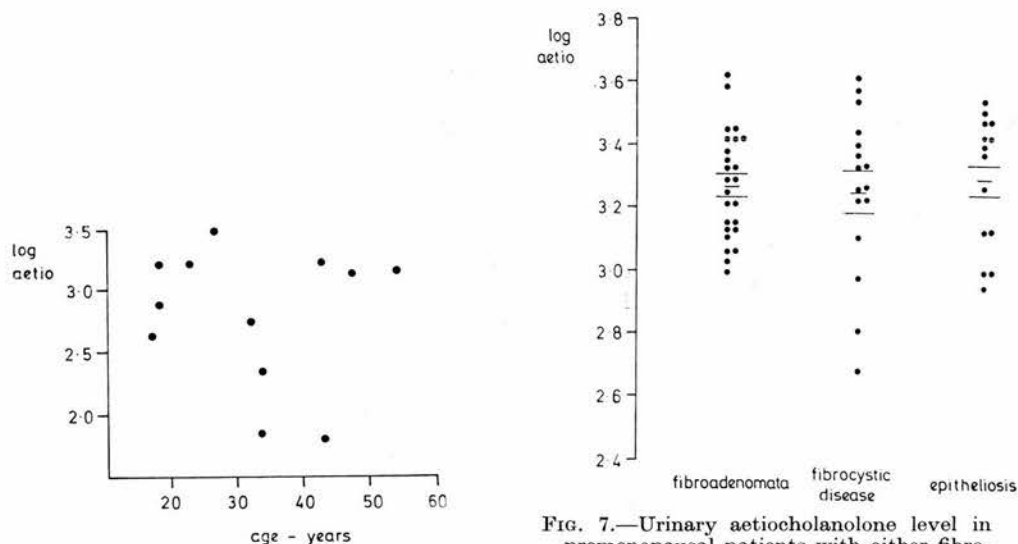


FIG. 6.—Scattergram of aetiocholanolone levels against age in 12 premenopausal control subjects from Cardiff.

FIG. 7.—Urinary aetiocholanolone level in premenopausal patients with either fibroadenomata, fibrocystic disease or epitheliosis of the breast (lines refer to mean \pm standard error for the groups).

cholanolone levels between benign, cancer and control patients, whether pre- or post-menopausal, in women from either Cardiff or Edinburgh. The further subdivision of the Edinburgh patients with benign breast disease into those with fibroadenomata, fibroadenosis or epitheliosis, revealed no differences between these groups (Fig. 7).

Correlation with other clinical and tumour parameters

Size, malignancy grade and round cell infiltration of the primary tumour, together with clinical palpability of lymph nodes, were assessed in 191 patients from the 2 centres. Histological examination of lymph nodes was performed on 120 patients. The partial correlation coefficients of aetiocholanolone with each of the other variables are shown in Table II.

TABLE II.—*Partial Correlation Coefficients of Aetiocholanolone and Variables of Possible Prognostic Significance*

	Cardiff (52 patients)	Edinburgh	
		Radical mastectomy (68 patients)	Total group (139 patients)
Age	-0.29	0.00	-0.02
Grade	0.30*	0.01	-0.19*
Round cell involvement	-0.30*	0.15	0.18*
Clinical involve- ment	0.11	0.02	0.01
Histological involvement	0.12	0.02	—
Size	0.22*	0.07	0.01

* $P < 0.05$.

These coefficients reflect the association between aetiocholanolone levels and each of these variables, after mutual association with menopausal status and with each of the other variables, has been taken into account (Steel and Torrie, 1960). In the Edinburgh series, the full set of partial correlation coefficients could only be obtained for the 68 patients

undergoing radical mastectomy, from whom histological examination of nodes was possible.

No consistent correlation was found between aetiocholanolone levels and any of these factors of prognostic significance. Significant but diverging relationships were, however, observed in Cardiff and in the full Edinburgh series with regard to both tumour grade and round cell infiltration. No obvious reasons are apparent for these paradoxical observations.

DISCUSSION

Using data obtained separately from the 2 centres, it has been possible to define the excretion of aetiocholanolone in control women and patients with benign and malignant breast disease, with respect to their age and menstrual status. By examining the data from patients with early breast cancer, it has also been possible to relate these aetiocholanolone levels to other factors known to be of prognostic significance.

The relationship between urinary aetiocholanolone and age has previously been expressed in the form of a quadratic equation (Cameron *et al.*, 1970), increasing age being associated with decreasing aetiocholanolone excretion. Whilst the present results are in accord with these findings, they also indicate that age *per se* is not associated with lowered aetiocholanolone levels. If the results are re-plotted after dividing the patients into pre- and post-menopausal groups, then age alone fails to influence urinary aetiocholanolone excretion significantly. It seems, therefore, that the reduction in aetiocholanolone observed with increasing age is a result of the increasing proportion of post-menopausal women. This relationship is also observed in control patients and in those with benign breast disease.

The present study, using a large number of patients, confirms the findings of Wade *et al.* (1969) and Cameron *et al.* (1970) that the mean urinary aetiocholanolone levels of patients with early

breast cancer are no different from those with either benign disease or in-patient controls. The observation that patients with epitheliosis (believed to be a pre-malignant condition) have normal aetiocholanolone levels does not support the suggestion that low aetiocholanolone levels may occur in women susceptible to breast cancer.

It has not been possible to demonstrate any consistent relationship between pre-operative urinary aetiocholanolone and any single one of a series of clinical and morphological factors of known prognostic significance in women with early breast cancer. These observations, therefore, are at variance with the suggestion that aetiocholanolone is of major prognostic significance in early breast cancer (Hayward and Bulbrook, 1968). In this respect, the time of the urine collection may be of importance. The data in the present study is based upon pre-operative specimens whereas those in Bulbrook's study related to urine collected 10 days after mastectomy. It is now known that mastectomy, possibly *via* the stress of surgical procedures, influences both urinary and plasma levels of androgenic steroids (Hayward and Bulbrook, 1968; Wang *et al.*, 1974). Conversely, patients awaiting surgery are likely to suffer pre-operative stress and this may also contribute to differences in pre- and post-operative steroid excretion.

It should be noted, however, that this study shows one group of patients, *i.e.* the post-menopausal women, who are likely to have low urinary aetiocholanolone levels. It is therefore interesting that pre-menopausal women over 35 years with breast cancer have a better prognosis than those women who are post-menopausal (Hamilton *et al.*, 1974). We therefore emphasize the importance of menopausal status in early breast cancer and suggest that it should be taken into account when assessing the possible predictive value of urinary aetiocholanolone.

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Urinary Etiocholanolone and Prognosis in Early Carcinoma of the Breast

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Etiocholanolone levels have been measured in both premastectomy and postoperative urines from 86 women with early breast cancer. Levels did not differ between patients subsequently surviving five years following mastectomy and those dying from recurrent disease within this period.

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Key words: etiocholanolone, carcinoma of the breast

INTRODUCTION

Evidence has been presented that, in patients with early breast cancer, etiocholanolone, especially in combination with corticosteroids, may predict disease recurrence and survival following mastectomy [Hayward and Bulbrook, 1968]. Others have claimed that levels of urinary androgens are without prognostic value for the patient with early disease [Alquist et al, 1968]. This conflict may arise in part by the fact that some investigators assayed preoperative urines while others have used postoperative samples [Hayward and Bulbrook, 1968]. In this paper we present results from both premastectomy and postoperative urines collected from patients with early breast cancer. The survival status of these patients five years after mastectomy is available and has been related to both pre- and postoperative levels of urinary etiocholanolone alone, and in combination with urinary corticosteroids as the discriminant function of Bulbrook et al [1960].

METHODS

The 86 patients studied formed part of the Edinburgh Breast Cancer Trial [Hamilton et al, 1974]. All had invasive, "operable" carcinoma of the breast and were of international clinical stages I and II, or were patients with stage III disease suitable for

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either of two treatment options: radical mastectomy or simple mastectomy plus radical radiotherapy. There were 23 premenopausal patients (experiencing regular menstrual periods), 54 postmenopausal patients (whose last period had occurred more than 2 years before treatment), and 8 menopausal patients (experiencing irregular periods or within 2 years of their last menstruation). The menopausal status of one woman was not recorded.

Twenty-four-hour urine collections were made on the day prior to mastectomy and postoperatively 7–10 days after operation. Urinary etiocholanolone levels were measured by the procedure described by Sneddon [1969] and 17-hydroxy corticosteroids by the method of Metcalfe [1963]. The discriminant function was calculated as described by Bulbrook et al [1960].

$$DF = 80 - 80 \times [17\text{-hydroxy corticosteroids (in mg)}] + \text{etiocholanolone (in } \mu\text{g)}$$

All statistical tests of significance were based on the logarithms of the etiocholanolone levels, and parametric tests were applied (analysis of variance, *t* tests).

RESULTS

Of the 86 patients 29 died within five years of mastectomy.

The preoperative and postoperative etiocholanolone levels for all patients are plotted in Fig. 1. There was a similar distribution for both surviving and nonsurviving patients. Most of the points in Figure 1 lie above the line along which the preoperative and postoperative values are equal, indicating that in the majority the urinary levels of etiocholanolone were lower after mastectomy.

Only 11 of the 47 women with stage I disease (23%) died within five years. This compared with 12 deaths among 23 women with stage II disease (52%) and 6 of 16 women with stage III disease (38%). Although mortality is dependent upon the stage of disease, the etiocholanolone levels showed no statistically significant differences between stages. This was true of preoperative levels, postoperative levels, and the percentage drop in level. Nor were there significant differences in etiocholanolone level between survivors and nonsurvivors within any stage.

Of the 23 premenopausal 5 women (22%) died within five years, compared to 5 of the 8 menopausal women (63%), and 19 of the 54 women who were postmenopausal (35%). There was no statistically significant difference at the 5% level between survivors and nonsurvivors in any menopausal group with regard to a) preoperative etiocholanolone level, b) postoperative etiocholanolone level, or c) change in etiocholanolone levels between pre- and postoperative urines (Fig. 2). In Figure 2c there is the suggestion of a consistently greater fall in etiocholanolone levels among nonsurvivors, but examination of an appropriately weighted mean difference by standard parametric methods failed to show statistical significance at the 5% level.

There were significant differences in the preoperative levels of etiocholanolone between the menopausal groups ($p < 0.01$), but neither the postoperative levels nor the drop in level demonstrated statistically significant differences between the menopausal groups ($p > 0.05$).

The mean levels of Bulbrook's discriminant function in various subgroups are given in Table I. The discriminant function is largely a reflection of the etiocholanolone level and the conclusions on the discriminant function are equivalent to results from the etiocholanolone data alone.

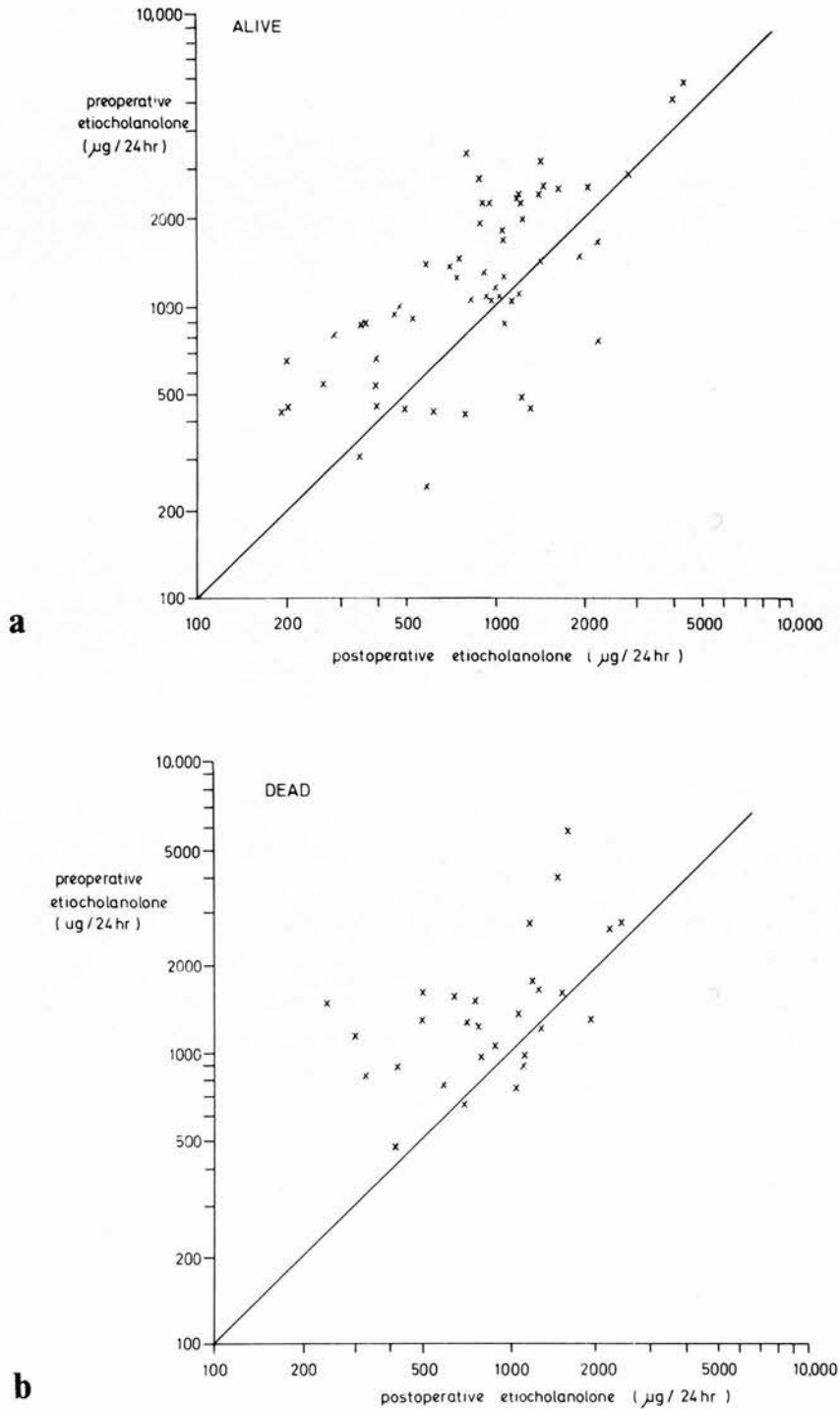


Fig. 1. Preoperative and postoperative levels of urinary etiocholanolone in women who were a) alive b) dead five years after mastectomy.

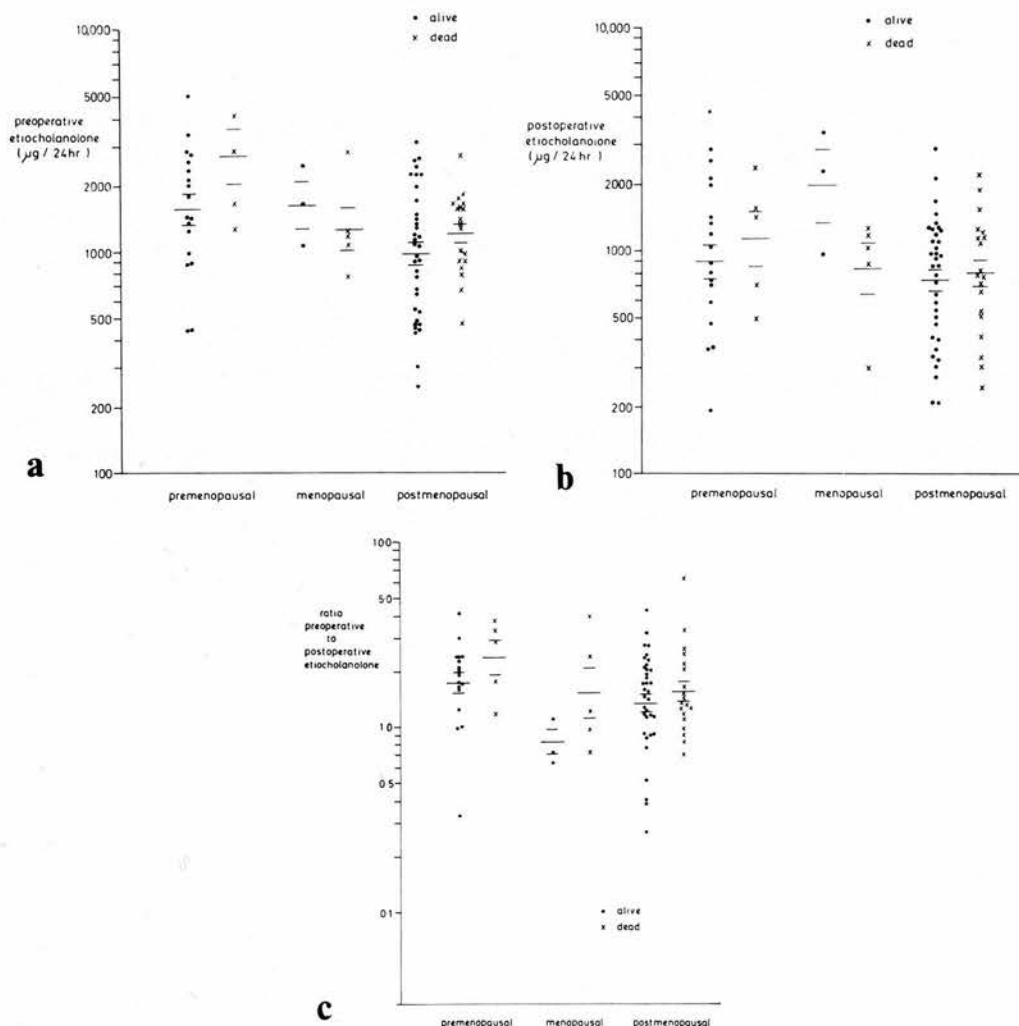


Fig. 2. Distribution of etiocholanolone levels in subgroups defined by menopausal status and five-year survival, with means \pm one standard error marked (log scale).

DISCUSSION

These results indicate no difference between survivors and nonsurvivors five years after mastectomy in the levels of urinary etiocholanolone. This conclusion was valid whether the measurements were performed on urine collected preoperatively or immediately postoperatively. Even combination with urinary corticosteroids (discriminant function) failed to distinguish between survivors and nonsurvivors. Accordingly, in our hands, etiocholanolone or discriminant values alone were of no assistance in forecasting survival after mastectomy.

It has been suggested, however, that etiocholanolone when combined with stage of disease, tumor grade, and menopausal status of the patients, may reveal a group of patients

TABLE I. Mean Values of Bulbrook's Discriminant Function (DF) (\pm Standard Error of Mean) in Subgroups of Patients Alive Five Years After Mastectomy and Patients in Corresponding Subgroups of Patients Dying Within Five Years of Mastectomy

Subgroup	Number of patients		Preoperative DF level		Postoperative DF level		Drop in DF	
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead
Stage								
I	36	11	1,000 (± 174)	708 (± 280)	636 (± 142)	360 (± 188)	363 (± 117)	347 (± 171)
II	11	12	1,267 (± 309)	1,334 (± 417)	555 (± 152)	623 (± 145)	713 (± 240)	711 (± 370)
III	10	6	608 (± 211)	1,061 (± 491)	274 (± 77)	420 (± 198)	334 (± 184)	641 (± 399)
Menopausal status								
Premenopausal	18	5	1,411 (± 258)	2,708 (± 857)	739 (± 190)	838 (± 389)	672 (± 156)	1,871 (± 697)
Menopausal	3	4	1,392 (± 421)	644 (± 511)	1,727 (± 718)	564 (± 62)	335 (± 297)	80 (± 497)
Postmenopausal	35	20	654 (± 127)	702 (± 129)	344 (± 85)	376 (± 106)	310 (± 104)	326 (± 97)

with poor prognosis [Hayward and Bulbrook, 1968]. While it is our experience that as a group postmenopausal patients have both lower urinary etiocholanolone and a poorer prognosis than premenopausal subjects [Hamilton et al, 1974], the addition of etiocholanolone to menopausal status and stage failed to improve prognostic accuracy. Furthermore, we have already shown that etiocholanolone does not appear to correlate with tumor parameters of possible prognostic significance [Miller et al, 1975].

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SECTION D
COMPOSITION OF BREAST FLUIDS



DEHYDROEPIANDROSTERONE SULPHATE IN BREAST SECRETIONS

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SUMMARY

Breast secretions obtained from non-lactating women by nipple aspiration contain high levels of material which cross-reacts with an antibody specific for dehydroepiandrosterone (DHA) and its sulphate. On thin layer chromatography, the cross reacting material migrates with DHA sulphate and after solvolysis with free DHA. The mass spectrum of the major component of solvolysed extracts after gas liquid chromatography (g.l.c.) also closely resembles that of authentic DHA. Quantitative estimates by g.l.c.-mass spectrometry of DHA in solvolysed extracts are similar to those obtained by radioimmunoassay of DHA and, after correction for recovery losses, compare well with radioimmunoassay values for DHA sulphate in the original secretions. It is concluded that breast secretions contain remarkably high concentrations of DHA sulphate.

INTRODUCTION

Breast secretions may be obtained from non-lactating women (both parous and nulliparous) by nipple aspiration [1]. Preliminary results have shown that these secretions may contain high levels of material which cross-reacts strongly with an antibody raised against DHA-3 β -monohemisuccinate conjugated to ovalbumin [2]. We now present evidence that this is DHA sulphate.

EXPERIMENTAL

Breast secretions

Breast secretions were collected by nipple aspiration using a suction cup as described by Sartorius [3]. Details of subjects and secretions are presented in Table 1. At the time of study all women had no history of breast abnormality except for subjects 3, 11 and 14 who presented with fibrocystic disease, and patient 12 who had carcinoma of the right breast.

Table 1. Details of secretions studied

Subject	Menopausal Status	Parity	Volume of Secretion (μ l)			
1.	Postmenopausal	Parous	L(a)	100	L(b)	85
2.	Premenopausal	Parous	L	40	R	170
3.	Menopausal	Parous	L(a)	40	L(b)	85
4.	Premenopausal	Parous	A†L	150	R	200
			B L	135	R	300
			C L	65	R	340
			D L	40		
5.	Postmenopausal	Parous	L	105		
6.	Premenopausal	Parous	R	40		
7.	Menopausal	Parous	L	45		
8.	Premenopausal	Parous	R	15		
9.	Premenopausal	Nulliparous	A L	35	R	310
			B L	40		
10.	Premenopausal	Parous	R	40		
11.	Premenopausal	Parous	L	80		
12.	Premenopausal	Parous	L	215	R	240
13.	Premenopausal	Parous	L	20		
14.	Premenopausal	Nulliparous	R	30		
15.	Premenopausal	—	L	20		

* L = Left Breast; R = Right Breast; a,b = different ducts in the same breast.

† A,B,C,D = Secretions collected on different occasions.

Table 2. Cross-reactivity of various steroids with antiserum used

Dehydroepiandrosterone sulphate	100%
Dehydroepiandrosterone	95%
Dehydroepiandrosterone glucuronide	70%
Epiandrosterone sulphate	70%
Epiandrosterone	9.5%
4-androsterone-3,17 dione	4.0%
Androsterone sulphate	2.5%
Androsterone	2.0%
Aetiocholanolone sulphate	0.15%
5 α Dihydrotestosterone	0.01%
Oestrone	0.01%
Testosterone	<0.01%
Testosterone sulphate	
Cholesterol	
Cholesterol sulphate	
3 β -hydroxy-5-pregnen-20-one	

All values are the mean of three determinations.

Cross-reaction calculated from ratio of molar mass of DHA sulphate to cross-reacting steroid, required to displace 50% of the radio-ligand, multiplied by 100.

Postmenopausal subjects were at least 2 years past their last menstrual period. Volume of secretion was measured by collection in calibrated capillary tubing. Secretions were stored diluted in phosphate buffer pH7 at -20°C . until assay.

Plasma

Peripheral blood was taken by venipuncture immediately before aspiration of breast secretions and collected in heparinized tubes. Plasma was separated within 30 min and stored at -20°C . until assay.

Radioimmunoassay

Radioimmunoassay of DHA sulphate was performed directly on diluted breast secretions and peripheral blood plasma using the method of Buster and Abrahams[4]. The antibody used was supplied by Mr B. A. Morris, Department of Biochemistry, University of Surrey and showed the specificity presented in Table 2. The same antibody was used to determine free DHA levels in solvolysed secretions using DHA to construct the standard curve.

Isotope tracer studies

[7 αH^3]-DHA sulphate (170,000 c.p.m., about 34 pmol) was added to aliquots of diluted breast secretions. The secretions were evaporated to dryness and the residue reconstituted in alcohol in preparation for thin layer chromatography (t.l.c.) on silica gel H.F. 254 + 366. Chromatography was performed in system I, isopropanol-chloroform-methanol-water (10:10:5:2 by vol.) for 2 h after which the area running with the mobility of authentic DHA sulphate was eluted with alcohol. The eluate was evaporated to dryness and solvolysed by dissolving in 0.05M sulphuric acid saturated with sodium chloride (5 ml). An equal volume of ethyl acetate was added and incubated overnight at 40°C . The ethyl acetate layer was taken off and the aqueous fraction re-extracted with

ethyl acetate. The bulked ethyl acetate fraction was evaporated to dryness and dissolved in alcohol, in preparation for thin layer chromatography. Chromatography was then performed in system II, chloroform-acetone (97:3 v/v) and the fraction migrating with authentic DHA eluted. Aliquots of secretion were taken for radioimmunoassay and estimation of radioactivity at the outset and after each chromatography step of the procedure.

G.l.c.-mass spectrometry

For identification purposes trimethyl silyl (TMS) derivatives of steroids and extracts were prepared [5]. Quantitative analysis was performed on tertiary butyl dimethyl silyl (TBDMS) derivatives using deuterated isoandrosterone as internal standard. This was prepared by hydrogenating DHA 3.5 (mmol) with platinum oxide catalyst in the presence of deuterium to give 5,6 dideutero isoandrosterone. This material (350 μmol) was equilibrated for 12 h with potassium hydroxide in D_2O and the steroids extracted with ether. After evaporation the residue was recrystallized from acetone/hexane to give 5,6,16,16 tetradeutero isoandrosterone. This standard was added to an aliquot of the solvolysed extract of breast secretion and dried down under nitrogen. The residue was derivatised as the TBDMS ether as described previously [6]. The steroids and internal standard were measured in a Dupont 490B mass spectrometer coupled through a glass jet separator to a Varian 1400 gas chromatograph. The gas chromatographic column used was a 10 m SE 30 support coated open tubular column (SGE Ltd) maintained at 210°C . The transfer lines were maintained at 250°C and the source temperature was 240°C . The steroids were quantitated by measuring the peak heights of the M-57 peaks (m/e 345 for DHA, m/e 347 for androsterone and isoandrosterone and m/e 349 for the tetradeutero isoandrosterone internal standard). Standard mixtures derivatised

alongside samples were used to construct a ratio-mass plot from which the mass of the various steroids present in the secretion was read. The intra-assay variation was 7.3% for androsterone, 20.5% for isoandrosterone and 9.5% for DHA. The limit of detection was 3.5 pmol.

RESULTS

Radioimmunoassay of breast secretions

All breast secretions studied contained high concentrations of material cross-reacting with the antibody to DHA-3B-monoheemisuccinate. Levels in secretions were between 50 and 350 fold higher than in plasma from the same patients (Table 3). Concentrations in secretions obtained from the same patient [4] on several occasions were remarkably consistent. Assay of secretions at different dilutions produced a dose response curve parallel to that of plasma and a standard curve of DHA sulphate (Fig. 1). After ether extraction, the antigen was still present in the aqueous fraction, less than 1% of the cross-reacting material being found in the organic phase.

After t.l.c. of the secretions in system I, material cross-reacting with the antibody was found in the area whose mobility corresponded to that of authentic DHA sulphate. No cross-reacting material was located in other areas including that corresponding to DHA glucuronide. After solvolysis of the antigen and t.l.c. in system II, the cross-reacting material migrated with authentic free DHA.

[7 α -³H]-DHA sulphate was added as tracer to three secretions and isotope tracer studies performed as described in the methods. The results are presented in Table 4 and it can be seen that the specific radioac-

Table 3. Concentration of DHA sulphate-like material (μ mol/l) as estimated by radioimmunoassay in breast secretions and plasma

Subject		Breast secretions	Plasma
1.	La	160	3.10
	Lb	260	
	R	322	
2.	L	416	2.44
	R	395	
3.	L(a)	867	5.03
	L(b)	846	
4.	A	L	5.37
		R	
	B	L	5.72
		R	
	C	L	4.33
		R	
	D	L	4.46
		R	
5.		L	2.48
6.		R	5.08
7.		L	3.10
8.		R	2.91
9.	A	L	9.00
		R	
	B	L	8.01
		R	
10.		R	6.07
11.		L	1.65
12.		L	1.70
		R	
13.		R	4.16
14.		R	—
15.		L	—

Legend as Table 1.

tivity as expressed as a ratio of ³H to cross-reacting material (in DHA or DHA sulphate equivalents) remained constant throughout chromatographic and solvolytic procedures.

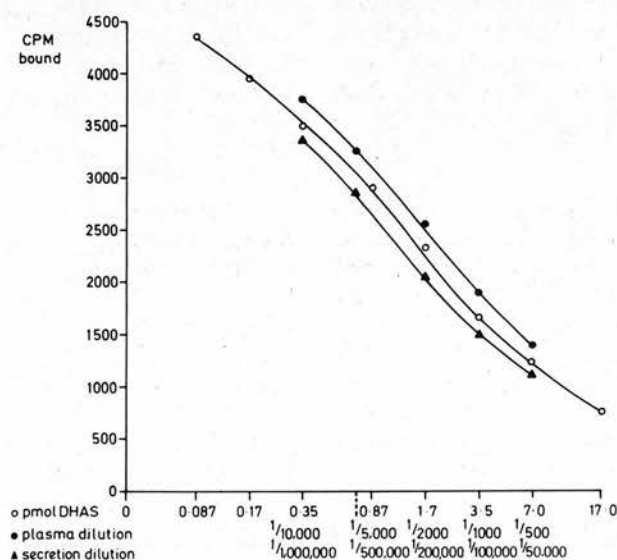


Fig. 1. Dose response curves using standard DHA sulphate (DHAS), breast secretion (3La) and plasma from the same patient (diluted as indicated and 0.5 ml assayed in duplicate).

Table 4. Isotope tracer studies of breast secretions

Secretion	Preparation	Total c.p.m.	Total nmol*	Specific radio-activity
13L	Diluted secretion	170,000	16.6	10,200
	After t.l.c. system I	121,500	12.1	10,000
	After solvolysis and t.l.c. system II	52,500	5.2	10,100
14R	Diluted secretion	170,000	12.1	14,000
	After t.l.c. system I	147,000	10.1	14,600
	After solvolysis and t.l.c. system II	66,000	4.6	14,300
15L	Diluted secretion	170,000	19.0	8,900
	After t.l.c. system I	117,500	10.3	11,400
	After solvolysis and t.l.c. system II	53,500	4.8	11,100

* As estimated from radioimmunoassay as DHA sulphate before solvolysis and DHA after solvolysis.

G.l.c.-mass spectrometry

Material isolated from the secretions after solvolysis and derivatisation to TMS ethers was subjected to g.l.c.-mass spectrometry. The largest component had a retention time identical to authentic DHA. When analysed by mass spectrometry, the spectrum of this constituent closely resembled that of authentic DHA-TMS ether (Fig. 2). The spectra showed a molecular ion at m/e 360, a peak at m/e 129 characteristic of TMS ethers of 3β -hydroxy- Δ^5 compounds [6] together with peaks at m/e 231 (M-129); there were other peaks corresponding to loss of trimethyl silanol (M-90) at m/e 270 and M-(90 + 15) at m/e 255. The peak at m/e 304 is probably due to the loss of C 15-17

from the steroid D ring. Several other g.l.c. peaks were present in solvolysed extracts and epiandrosterone and androsterone were identified by their retention times and mass spectra.

Quantitation of DHA, androsterone and epiandrosterone by g.l.c.-mass spectrometry of TBDMS ethers was performed in solvolysed extracts of 20 secretions and results are presented in Table 5. In each case DHA represented the major steroid identified although variable amounts of androsterone and epiandrosterone were also present. Analysis of ether extracts of pre-solvolysis secretions failed to demonstrate significant amounts of either free DHA, androsterone or epiandrosterone.

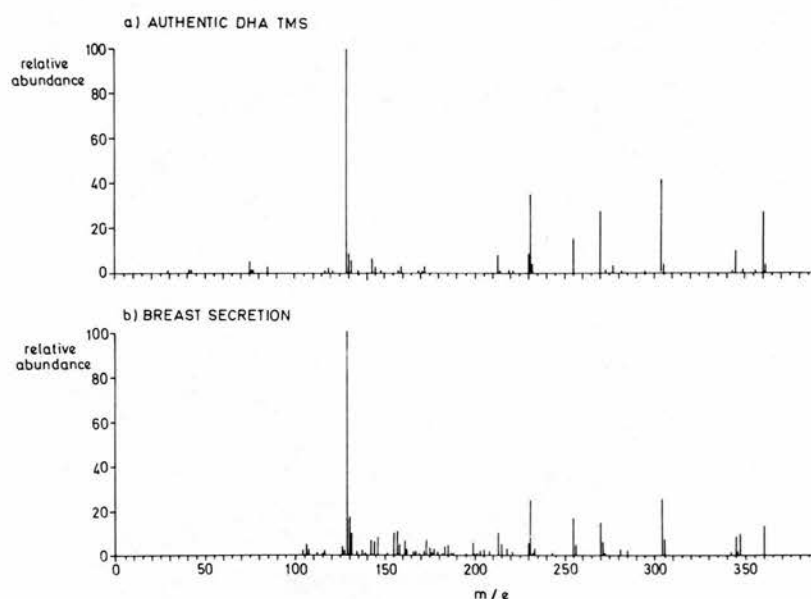


Fig. 2. Comparison of mass spectrometry of authentic DHA and solvolysed sample of breast secretion both derivatized as TMS ethers.

Table 5. G.l.c.-mass spectrometric analysis of solvolysed extracts of breast secretions

Secretion	Content (nmol)			
	By radioimmunoassay DHA	By g.l.c.-mass spectrometry		
		DHA	androsterone	epiandrosterone
1 L(a)	6.24	3.29	2.41	2.51
L(b)	10.05	7.28	3.96	0.90
R	1.39	1.35	0.86	0.76
2 R	22.35	17.15	2.69	5.03
L	6.93	7.84	3.27	7.30
3 L(a)	19.95	24.35	4.89	3.37
L(b)	57.20	54.90	7.58	2.07
4AL	88.40	56.30	11.00	5.99
R	88.40	81.60	11.90	6.68
BL	84.60	72.45	12.35	12.80
R	95.70	98.80	14.90	15.30
CL	32.25	28.10	5.51	5.51
5 L	25.40	21.85	3.65	2.75
6 R	59.70	49.95	4.34	2.75
7 L	15.95	18.05	1.83	0.48
8 R	9.02	9.36	2.75	1.17
9AL	36.75	36.95	8.95	10.35
10 R	17.50	16.65	2.96	5.27
11 L	5.13	4.15	1.38	2.75
12 L	27.75	24.25	6.40	5.51

Legend as Table 1.

Comparison of levels as measured by radioimmunoassay and g.l.c.-mass spectrometry

In solvolysed extracts of 20 secretions, DHA levels were measured by both radioimmunoassay and g.l.c.-mass spectrometry. In each case, levels detected by both methods were of a similar order of magnitude (Table 5) and the correlation about a line of linear regression was highly significant at $P < 0.001$ (Fig. 3).

Losses during solvolysis were determined in 10 secretions by measuring the recovery of added tracer [$7\alpha^3\text{H}$]-DHA sulphate (20,000 c.p.m., about 4 pmol). It was thus possible to calculate DHA sulphate levels in the original secretions from g.l.c.-mass spectrometric estimates of DHA in the solvolysed extract. These have been compared with values for DHA sulphate obtained by radioimmunoassay performed directly on diluted secretions (Fig. 4). Correlation of estimates of DHA sulphate by the two methods is highly significant ($P < 0.001$).

DISCUSSION

We have previously reported that breast secretions from nonlactating women contain large amounts of material cross reacting with an antibody largely specific for DHA and its sulphate [2]. Details of further characterization of the material is now presented. The cross-reacting material is not ether extractable and moves with the mobility of authentic DHA sulphate during thin layer chromatography. However, once solvolysed the mobility changes to that of free DHA. If [$7\alpha^3\text{H}$]-DHA sulphate is added

to secretions as tracer, the ratio of radioactivity to cross reacting material remains constant throughout these procedures. g.l.c.-mass spectrometry analysis of the solvolysed material confirms the identity of DHA; variable amounts of epiandrosterone and androsterone are also present. The levels of DHA in solvolysed extracts are similar whether estimated by radioimmunoassay or g.l.c.-mass spectrometry. Although the mass-spectrometric data relates to DHA in solvolysed extracts, significant amounts of free steroid are unlikely to have been present before solvolysis as all secretions were extracted with ether at the outset of characterization. After chromatography in t.l.c. system I which separated DHA sulphate from DHA glu-

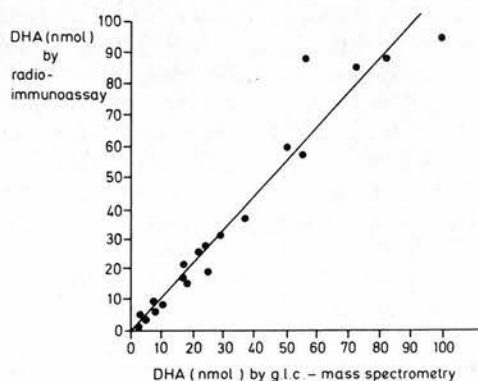


Fig. 3. Correlation between DHA content in solvolysed extracts of breast secretions as estimated by radioimmunoassay and g.l.c.-mass spectrometry. Regression line $y = 1.09x + 0.922$ correlation coefficient $r = 0.972$.

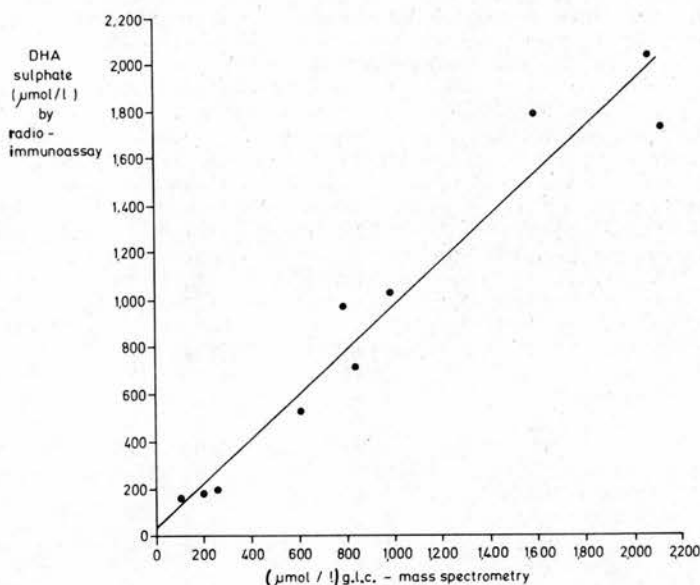


Fig. 4. Correlation between DHA sulphate concentrations in breast secretions as assayed by direct radioimmunoassay and calculated from g.l.c.-mass spectrometric estimates of DHA in solvolysed extracts. Regression line $y = 0.933x + 54.8$. Correlation coefficient $r = 0.973$. Breast secretions examined were 4CL, 4CR, 4DL, 9AL, 9AR, 9BL, 10R, 11L, 12L and 12R.

curonide, no cross reacting material was found with a mobility of DHA glucuronide. Furthermore, calculation of DHA sulphate levels in secretions from g.l.c.-mass spectrometric determination of DHA in their solvolysed extracts (after correction for procedural losses and molecular weight changes) agrees well with values for DHA-sulphate obtained directly by radioimmunoassay—even in those secretions subject to chromatography before solvolysis.

This data provides conclusive evidence that breast secretions may contain remarkably high levels of DHA sulphate. Values may be as much as 300 fold higher than in plasma from the same woman. Although it has been reported that breast cyst fluids may contain high levels of androsterone and DHA sulphate [7] and oestrogen conjugates [8], we believe this is the first report of high levels of C19 steroid conjugates in breast secretions. Raised levels of other hormones have been reported in breast secretions [9] but in absolute amounts, these are small compared with the levels of DHA sulphate detected in this study. Similarly although levels of progesterone may be high in perinatal mammary secretions from women [10] and cow's milk during early pregnancy [11] the values are 10-100 fold less than those of DHA sulphate in breast secretions from non-pregnant women.

At the present time it is not possible to state whether the high levels of DHA sulphate in breast secretions are due to active synthesis by the breast or the result of concentration from plasma sources. However, it has been reported that the amounts of steroid conjugates transferred from plasma to milk

are minimal [12] although this may not be relevant as levels of DHA sulphate in human milk are low (Miller, W. R., unpublished results). The physiological implications of elevated levels of DHA sulphate in breast fluid remain to be elucidated but breast tissues are capable of utilizing DHA sulphate as a precursor for more powerful hormones such as testosterone [13] and plasma levels of DHA sulphate may differ in women with varying risk to breast cancer [14].

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Factors affecting dehydroepiandrosterone sulphate levels in human breast secretions

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Keywords: androgen, breast secretion

Summary

Human breast secretions as collected by nipple aspiration have been analysed for dehydroepiandrosterone sulphate by radioimmunoassay. All secretions collected from non-lactating normal women contained remarkably high levels of dehydroepiandrosterone sulphate as compared with plasma taken at the same time. Although there was a large range of concentrations, levels were of a similar magnitude in different ducts from the same breast and in different breasts from the same individual. No significant difference was detected between secretions from pre and postmenopausal women. Sequential sampling of breast secretions through the menstrual cycle in two normal premenopausal women showed no cyclic variation in dehydroepiandrosterone sulphate concentration. There was also no significant difference between levels in breast secretions obtained from normal women and patients with either malignant or benign breast disease. Analysis of secretions from tumour and non-tumour bearing breasts in cancer patients failed to show consistent differences although, in contrast to normal women, the variation between breasts in individual patients was often marked.

Introduction

Information on the steroid hormone content of human non-lactating breast secretions is limited. A report that free oestrogens are present in higher concentrations than in plasma (1) was not confirmed in our laboratory (2). On the other hand we found that breast secretions contained large amounts of material cross reacting with an antibody specific for dehydroepiandrosterone and its sulphate (2) and subsequently characterised this as dehydroepiandrosterone sulphate by GLC-mass spectrometry (3). We now describe our findings on a large series of secretions obtained from both normal women and patients with breast disease.

Materials and methods

Subjects

Breast secretions were obtained from 53 of 90 women studied at random. There were 22 normal

women, 7 with fibrocystic disease, 2 with breast pain and 22 with breast cancer. Twenty-eight women were premenopausal, 14 postmenopausal (>two years past their last menstrual period), 9 menopausal and 2 of unknown menstrual status. The date of the last menstrual period was noted for all premenopausal women.

Breast secretions were collected by aspiration using a nipple cup as described by Sartorius (4). These were stored, diluted between 100 and 500 fold in 0.2M phosphate buffer pH 7.0, at -20°C. In each woman, attempts were made to collect secretions from both breasts but this was only possible in 34; in 9, secretions were obtained from the left breast alone and in 10 from the right breast alone. Four women were studied on more than one occasion. A total of 122 secretions were collected with volumes varying between 10 and 250 µl.

Peripheral blood was taken immediately before aspiration of breast secretions, collected in heparinised tubes, centrifuged and the plasma stored at -20°C.

Radioimmunoassay

Radioimmunoassay of dehydroepiandrosterone (DHA) sulphate was performed directly on diluted breast secretions and peripheral blood plasma using the method of Buster and Abraham (5). The specificity of the antibody has been described previously (3).

Results*Normal women*

Sixty-seven secretions were collected from 22 normal women. The mean DHA sulphate concentration in breast secretions and plasma for these subjects is shown in Table 1.

In 15 women, secretion was obtained from both right and left breasts. The results are presented in Fig. 1. Concentrations of DHA sulphate were comparable in secretions from different breasts in the same individual.

In two women, secretion was obtained from different ducts in the same breast. These results are shown in Table 2 and indicate that DHA sulphate levels were again of similar magnitude.

Because of the similarity of values in secretions from the same women, in the following comparisons, results of multiple secretions from the same

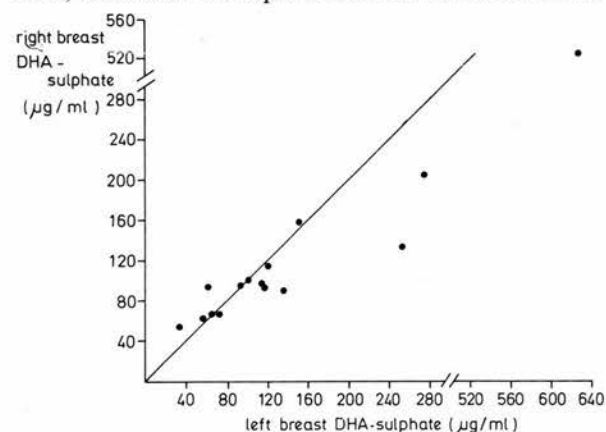


Fig. 1. DHA sulphate levels in secretions from both breasts of normal women. The line is that on which points should fall if concentrations were identical in both breasts. Where the same woman was studied on more than one occasion, values for each breast have been averaged.

Table 1. Levels of DHA sulphate ($\mu\text{g/ml}$) as measured by radioimmunoassay in breast secretions and plasma from twenty-two normal women.

	Breast secretions (67) ^a	Plasma (35) ^a
Mean	250	1.27
Range	(8.60–575)	(0.10–2.94)

^a Sixty-seven secretions and 35 plasma samples were obtained from twenty-two normal women – certain subjects were studied on more than one occasion.

Table 2. Comparison of DHA sulphate concentration ($\mu\text{g/ml}$) in secretions from separate ducts in the same breast.

Subject	EMcA	RMcQ
Left breast duct 1	195	287
duct 2	112	218
Right breast duct 1	128	156
duct 2	172	—
duct 3	122	—

individual have been averaged.

No relationship was apparent between DHA sulphate levels in secretions and age of the women (Fig. 2) nor was there a significant difference between secretions from pre and postmenopausal women (Fig. 3). It was noticeable however, that all

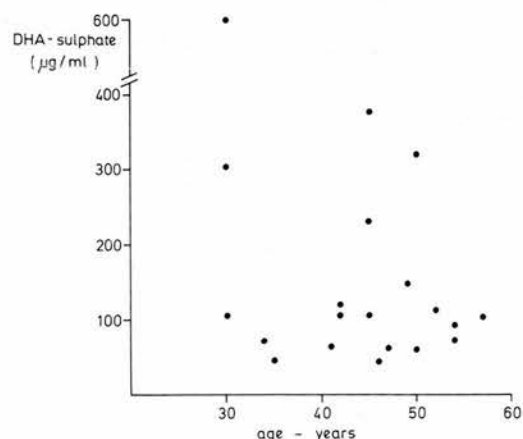


Fig. 2. The relationship between DHA sulphate levels in breast secretions and age in normal women. Where more than one secretion was obtained from an individual, the values have been averaged.

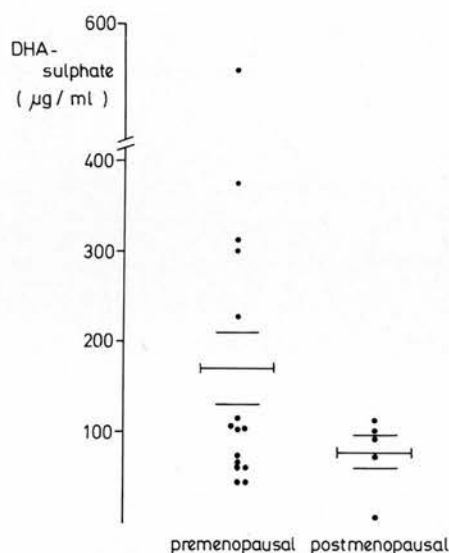


Fig. 3. Levels of DHA sulphate in breast secretions from pre and postmenopausal normal women. Lines are mean and standard error of the mean. Where more than one secretion was obtained from an individual, the values have been averaged.

secretions with values in excess of 200 $\mu\text{g/ml}$ came from premenopausal women and the number of postmenopausal women studied was small.

The effect of sequential sampling through the menstrual cycle on DHA sulphate concentrations in breast secretions has been studied in two women. Both had regular menstrual periods and no history of taking oral steroids, no family history or past history of breast disease and no clinical or radiographic breast abnormality. One subject (R) was aged 45 years and multiparous, the other (E) was aged 30 years and nulliparous. Secretions were taken from both breasts on seven occasions through consecutive menstrual cycles (at about seven day intervals for subject R and five day intervals for subject E). Between 10 and 250 μl of secretion was collected on each occasion but there was no obvious cyclic change in volume or total protein content of secretion. Levels of DHA sulphate in secretions and peripheral plasma are shown in Fig. 4. Throughout the cycle there was remarkable high concentrations of DHA sulphate in secretions compared with plasma (secretion 100–250 fold higher than plasma) and in each patient, concentrations in secretions from the left breast were always higher than those from the right. There was, however, no obvious

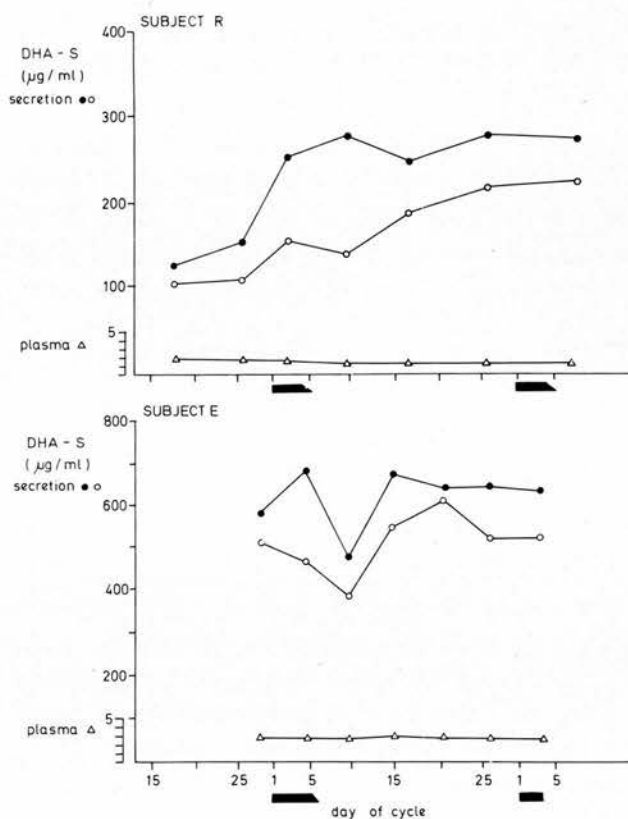


Fig. 4. Levels of DHA sulphate in breast secretions and plasma taken from 2 normal premenopausal women through the menstrual cycle. ● Secretion from left breast, ○ secretion from right breast, Δ plasma.

cyclic pattern in DHA sulphate levels in secretions or plasma during the menstrual cycle and no evidence for a decrease in concentration in secretions with continued sampling.

Women with breast disease

Levels of DHA sulphate in secretions from women with benign and malignant breast disease are compared with those from normal women in Table 3 and in Fig. 5. No significant differences were detected and, like normal subjects, most women with benign and malignant disease had levels in secretions many times higher than in their plasma (Fig. 6).

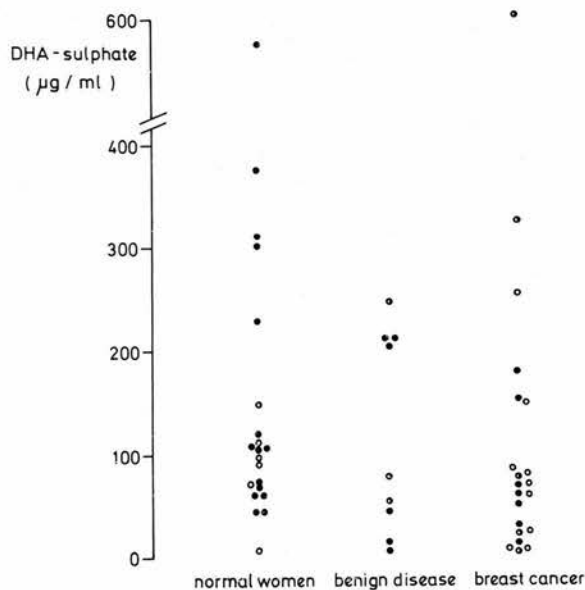
In thirteen women with breast cancer, secretions were obtained from both breasts so that comparison between tumour bearing and non-tumour

Table 3. Levels of DHA sulphate ($\mu\text{g/ml}$) in secretions from normal women and patients with benign or malignant breast disease.

	Normal (22)	Benign (9)	Breast cancer (22)
Mean \pm SEM	149 \pm 30	119 \pm 33	109 \pm 33
Range	9–575	6–247	1–678

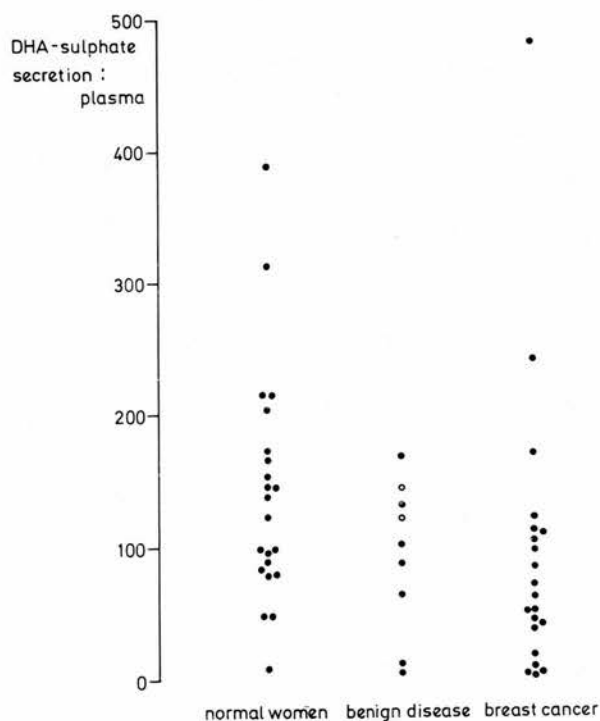
Figures in parentheses represent the number of patients studied. Where more than one secretion was obtained from an individual, values for each were averaged before calculation of the means for the group. This accounts for the difference in mean for the normal group as presented in Table 1 which was calculated by directly meaning the values for the total number of secretions collected from normal women.

bearing breasts could be made (Table 4). Although in about one half of patients there were large variations in DHA sulphate levels between breasts of the same individual, in some cases the tumour bearing breast gave secretions with higher concentrations and in others lower concentrations of DHA sulphate.

**Fig. 5.** DHA sulphate levels in breast secretions from normal women, patients with benign breast disease and those with breast cancer. Where more than one secretion was obtained from an individual, the values have been averaged. ● premenopausal, ◐ menopausal, ○ postmenopausal.**Table 4.** Levels of DHA sulphate ($\mu\text{g/ml}$) in secretions from tumour bearing and non-tumour bearing breasts of women with breast cancer.

Subject	Tumour bearing	Non-tumour bearing
1.	155.0	140.0
2.	60.0	62.0
3.	1.6	0.5
4.	3.2	3.5
5.	19.2	30.5
6.	18.4	118.0
7.	57.0	44.0
8. ^a	(a) 456.0 (b) 216.0	320.0
9.	76.0	64.0
10.	34.5	34.0
11.	200.0	111.0
12.	1.9	17.6
13.	21.5	4.3

^a Secretions were obtained from two separate ducts (a and b).

**Fig. 6.** Ratios of DHA sulphate between breast secretions and plasma in individual normal women and patients with either benign breast disease or cancer. Within the benign disease group ● fibroadenosis or fibrocystic disease, ○ mastalgia.

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TABLE 1

IgA, IgG and lactoferrin in breast secretions from healthy non-lactating subjects, post-partum colostrum and milk

Subject No.	Age	Menstrual states	Parity	Secretion No. ^c	IgA concn. (g/l)	IgG concn. (g/l)	Lactoferrin concn. (g/l)
1	42	Premenopausal	Not known	19R	114.73	2.50	—
2	52	Postmenopausal	Parous	20L ₁ 20L ₂ 20R	122.78 116.98 125.80	2.81 2.89 3.26	— — —
3	49	Menopausal	Parous	23L ₁ 23L ₂ 23R	6.00 4.52 10.82	1.28 1.45 1.91	— — —
4	Not known	Postmenopausal	Not known	24L	160.50	67.08	—
5	45	Premenopausal	Parous	32L 32R 37L ₁ 37L ₂	144.15 144.20 108.10 237.60	25.90 1.08 1.26 2.02	2.1 1.3 2.2 n.d.
6	30	Premenopausal	Nulliparous	37R 34L 34R	108.80 104.90 126.60	1.05 1.11 1.04	1.05 n.d. 120.0
7	35	Premenopausal	Not known	38 35 39R	181.70 57.80 234.20	1.22 6.15 7.91	n.d. n.d. n.d.
Colostrum ^a					120.47	0.34	14.82
Day 6 milk ^a					3.68	0.04	4.95
Serum ^b					1.24	5.69	n.d.

^a Data from McClelland et al. (1978), from mean colostrum and milk concentrations ($n = 53$ and 31 , respectively).^b Unpublished observations by McClelland and Samson ($n = 19$). Serum IgA was determined using a 7S IgA standard.^c The secretion number indicates the way in which the secretion was obtained: e.g. for patient No. 5, 32L and 32R were taken from the left and right breasts, respectively, on the same occasion; on another occasion, 37L₁, 37L₂ and 37R were obtained, but the secretion within two separate ducts ending in the left nipple (L₁, L₂) were collected individually.
n.d. = not detected.

Concentrations of 11S IgA and IgG in breast secretions

11S IgA and IgG were detectable in all the breast secretions obtained from normal subjects; values range from 4.5 to 237.6 g/l and 1.4 to 67.1 g/l, respectively. The mean (\pm S.E., $n = 18$) 11S IgA concentration of 117.2 ± 15.3 g/l was significantly higher than the mean IgG concentration of 7.3 ± 3.7 g/l ($P < 0.001$; unpaired t -test), but no significant correlation existed between 11S IgA and IgG values for each individual secretion ($r = 0.2204$, $P < 0.40$; Table 1).

In an attempt to assess the validity of the immunoglobulin concentrations observed in breast secretions, the value for the right breast was compared with the value obtained for the left breast, where secretions were obtained from both breasts on the same occasion. Where more than one secretion was obtained from a breast on a particular occasion (e.g. subject 5, secretions 37L₁, 37L₂), the mean value for the breast was used for the comparison. IgA values for the right breast ranged from 63 to 206% of the values seen for the left breast (mean \pm S.E. = 119 ± 24 ; $n = 5$) while corresponding IgG values showed a greater degree of variation (due to the IgG value for secretion 32L, subject 5) ranging from 4 to 114% (mean \pm S.E. = $67 \pm 18\%$). In three subjects (Nos. 2, 3 and 5) the secretion obtained from each of two different ducts within the same breast was assayed separately. 11S IgA and IgG concentrations were very similar in two subjects (Nos. 2 and 3) but showed a two-fold variation in the third (subject No. 5), suggesting that a variation in the immunoglobulin concentration of secretions might occur between ducts in the same breast (Table 1).

In view of the small number of subjects studied, and the incomplete clinical data, it was not possible to assess the relationship of age, menstrual status or parity to immunoglobulin concentrations of breast secretions. However, high concentrations of 11S IgA were found in breast secretions in all the subjects studied, particularly in the secretions obtained from postmenopausal or nulliparous subjects (Nos. 2 and 4; No. 6). Concentrations of 11S IgA were in general very similar to the range of concentrations seen in postpartum colostrum and milk (McClelland et al., 1978). As in colostrum and milk, the ratio of IgA present in the breast secretions from healthy subjects to IgA present in serum was very much greater than the corresponding ratio for IgG (Table 1). The reason for the high concentrations of IgG detected in the secretions of subjects 4 and 5 is not clear.

Among the small number of secretions studied from subjects with breast disease, all but two of the secretions (31L, 33L) were within the range of 11S IgA concentrations found for secretions for normal subjects. Similarly, all but two secretions (15R, 30R) were within the range of IgG concentrations seen in the secretions of normal subjects. This suggests that no major differences are likely to be present between breast secretions obtained from normal subjects and those obtained from patients with breast disease (Table 2).

Fig. 2. Gel filtration on a Sepharose 4B column (2.2×90 cm) of (a) mammary secretion obtained from a healthy non-lactating woman (b) colostrum (●) and milk (○) and (c) normal adult serum (X). 3-ml fractions were collected and assayed by radioimmunoassay using an 11S IgA standard (colostrum, milk and mammary secretion) or a 7S IgA standard (normal adult serum); antisera used were either rabbit anti- α chain (indicated in a by ●) which detects all forms of IgA, or anti-11S IgA/FSC (used only in a and indicated by ○) which detects only 11S-IgA and free secretory component. The arrow indicates the elution volume of a Blue Dextran marker (B.D.: void volume).

Concentrations of lactoferrin in breast secretions

Concentrations of lactoferrin similar to the concentrations found in colostrum and milk were detected in 5 out of 10 breast secretions obtained from three normal subjects (Nos. 5, 6 and 7). The failure to detect lactoferrin in the remaining secretions was probably due to the dilution (1/200 to 1/500) of the secretion before assay.

DISCUSSION

In the breast secretions obtained from healthy non-lactating women the IgA was 11S as indicated by ^{125}I -labelled 11S IgA binding inhibition curves and gel filtration analysis. This was confirmed using anti-11S IgA/FSC which indicated that it was the complete 11S IgA molecule that was detected, and not the 10S form. Interestingly, high molecular weight forms of IgA ($>400\,000$ daltons) were also detected by gel filtration, as was demonstrated in early colostrum (Wadsworth, 1978). This IgA could possibly represent aggregation of 11S IgA or the synthesis of high molecular weight forms ($>11\text{S}$). Very little IgA immunoreactive material eluted at the same volume as 7S IgA.

Absolute concentrations of IgA and IgG in normal breast secretions are very similar to the values found in colostrum and milk (McClelland et al., 1978). The ratio of IgG in normal breast secretions to IgA in serum was much higher than the corresponding ratios of IgG, indicating that the 11S IgA found in normal breast secretions was due to selective transport or local synthesis. As there is little 11S IgA in adult serum (Waldman et al., 1970), the 11S IgA detected must be a product of local synthesis. This confirms the earlier report of Drife et al. (1976), who detected IgA synthesis in 81% of cultures of normal human breast tissue.

In the present study, IgA and IgG were detected in all samples from both normal and diseased breasts, with a wide range of concentrations observed. In contrast, Petrakis et al. (1977), reported the absence of IgA and IgG in a number of normal breast secretions and secretions from cancerous breasts, with mean concentrations seventeen and three times lower, respectively, than in our study. This difference may be attributed to the use of a less sensitive method of detection of immunoglobulins (rocket immunoelectrophoresis), to the possible use of a standard different from our 11S IgA standard (Schuurman, 1977; Greenberg et al., 1978), or because their assays were performed on undiluted samples. Our results would suggest that if a deficiency exists it is a relative rather than an absolute one.

Although high concentrations of 11S IgA were detected in the normal breast secretions the volume of the secretions was very small and hence the total 11S IgA synthesis by the non-lactating breast is very much less than that of the lactating breast. Only a fraction of the total secretion lying within the mammary ducts of the non-lactating breast is obtained by aspiration with the suction cup, and the values for IgA, IgG and lactoferrin observed should therefore be considered as being representative of the ductal secretion nearest the nipple, rather than of the entire breast. For the secretions obtained from normal subjects on a particular occasion (whether from left or right breast), IgA concentrations with respect to each individual patient vary up to two-fold. IgG concentrations vary similarly with the exception of secretion 32L. This suggests that measurements of immunoglobulin concentrations in breast secretions may be a valid procedure despite the problem of obtaining uniform volumes of secretion.

and that the amount of these steroids in cyst fluid is not correlated with the volume of the cyst. The results of the present study would confirm these findings. Most cyst fluids contained large quantities of DHAS, but there was an extensive range of concentrations present in different cysts. In addition, GLC-MS confirmed the presence of large amounts of androsterone conjugate. Epiandrosterone conjugates were also present, and their high concentration probably accounts for the inconsistencies sometimes observed between the methods of radioimmunoassay and GLC-MS in the estimation of DHAS in cyst fluids. The antibody used in the radioimmunoassay cross-reacts significantly with epiandrosterone sulfate (2). It thus seems likely that in some cyst fluids, results from radioimmunoassay represent not only DHAS but also a contribution from other closely related steroid conjugates.

Because of the broad range of DHAS found in cyst fluids, it was of interest to examine factors that might influence these levels. Although most cyst fluids were obtained from premenopausal patients, no account was taken of the stage of the menstrual cycle in which they were collected. No cyclic pattern has been observed for DHAS levels in either plasma (6) or breast secretions (7). We could detect no significant correlation between cyst fluid concentration of DHAS and age, menopausal status, or parity of the patients. A similar lack of correlation has been reported by Bradlow et al. (5).

In a small number of cases we were able to analyze several cysts from the same women. There was a good correlation between DHAS levels in cysts aspirated from the same breast at the time, but larger variations were apparent between cysts from different breasts of the same women even when these were sampled at the same time. Cysts aspirated from the same women on sequential visits showed broad differences in DHAS concentrations, even though they may have been derived from the same breast. Similar observations have been made by others (7). The widely different values that may be observed in cysts aspirated from the same breast on separate occasions and, indeed, from both breasts of the same women when studied at the

same time, makes comparative studies between different groups of women very difficult. It will be necessary to discover why such variations exist and the mechanism by which steroid conjugates accumulate in breast cysts. They may be derived from the breast secretory units or represent a transudate from the circulation. It is thus significant that breast secretions obtained by nipple aspiration also contain high levels of DHAS. Our limited comparison of breast secretions and cyst fluids obtained from the same women suggests that breast secretions invariably contain the higher concentration. It therefore seems likely that certain cyst fluids represent accumulation and dilution of breast secretions by blockage of the ducts in which they reside. Our observation (8) that cyst fluids with high DHAS concentrations contain only low levels of albumin, whereas those with low DHAS have high concentrations of albumin would support this contention and suggest that the diluent is plasma derived. It may be necessary to subdivide cyst fluids according to whether the derivation of their major constituents is breast or plasma before meaningful studies on their composition can be performed.

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Androgen Conjugates in Human Breast Secretions and Cyst Fluids

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Despite many years of research, it has not been possible to demonstrate conclusively that risk or presence of breast disease is associated with specific hormonal abnormalities. However, most studies have measured hormones excreted in urine or circulating in blood. Such measurements may not reflect the endocrine environment within the breast itself. In order to obtain a better estimate of the milieu within the breast, we have measured hormones in breast fluids. This chapter is concerned with levels of dehydroepiandrosterone (DHA) sulfate and other related androgen conjugates in human breast secretions and cyst fluids.

METHODS

Breast secretions were obtained by nipple aspiration, applying a slight negative pressure by means of a syringe attached to a nipple cup (5). In this way, 132 breast secretions were obtained from 51 nonlactating women. (Where possible, secretion was obtained from both breasts of the same woman, and some subjects were studied on more than one occasion.) The women comprised 21 normal subjects who had no evidence of breast abnormality, 9 patients with benign breast disease, and 21 with breast cancer.

Cyst fluids were obtained by needle puncture of 100 tension cysts of the breast in 82 different women. Eight patients had multiple cysts, and 5 were studied on more than one occasion.

Radioimmunoassay was performed directly on all breast fluids after dilution with phosphate buffer, using the method of Buster and Abraham (1) and the antibody described previously (3). DHA-sulfate was also measured by gas-liquid chromatography (g.l.c.)-mass spectrometry in 20 breast secretions and 6 cyst fluids (3). Results from the two methods were comparable.

In measuring androsterone and epiandrosterone conjugates, all estimations were performed by g.l.c.-mass spectrometry (3).

RESULTS AND DISCUSSION

Breast Secretions

The mean value for DHA-sulfate in the 132 breast secretions investigated was 345 $\mu\text{mol/l}$ (Table 1). This was more than 100-fold higher than that in plasmas

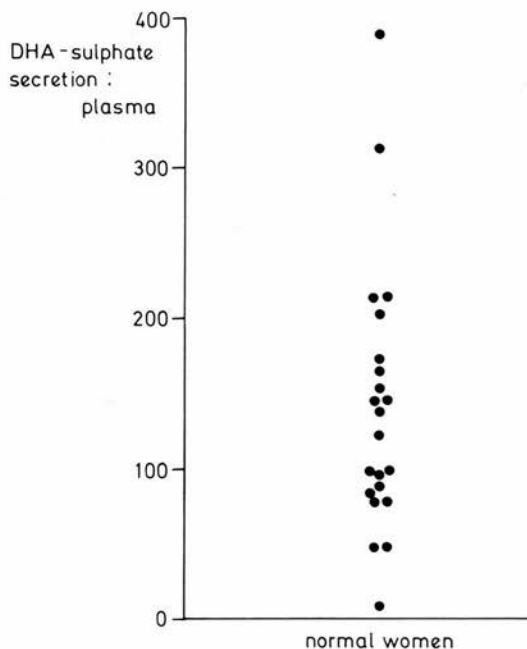


FIG. 1. Breast secretion:plasma ratios of DHA-sulfate in 21 normal women. Where more than one secretion was obtained from a subject, the values have been averaged before expression as a ratio.

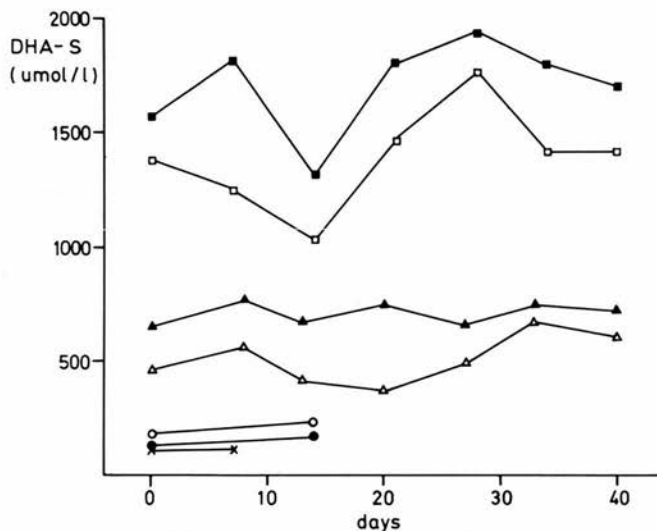


FIG. 2. DHA-sulfate concentrations in breast secretions obtained from the same individuals on different occasions. Day 0 represents the first occasion on which secretion was obtained. ● ▲ ■ x represent secretions from left breast of different women; ○ △ □, right breast.

TABLE 1. Levels of DHA-sulfate ($\mu\text{mol/l}$) as measured by radioimmunoassay in breast secretions and plasmas from 51 women studied

	Breast secretions ^a	Plasmas ^a
Mean	345	3
Range	5-2,000	0.5-10

^a 132 breast secretions and 63 plasmas were obtained from 51 women. Certain subjects were studied on more than one occasion, and secretions were often obtained from both breasts.

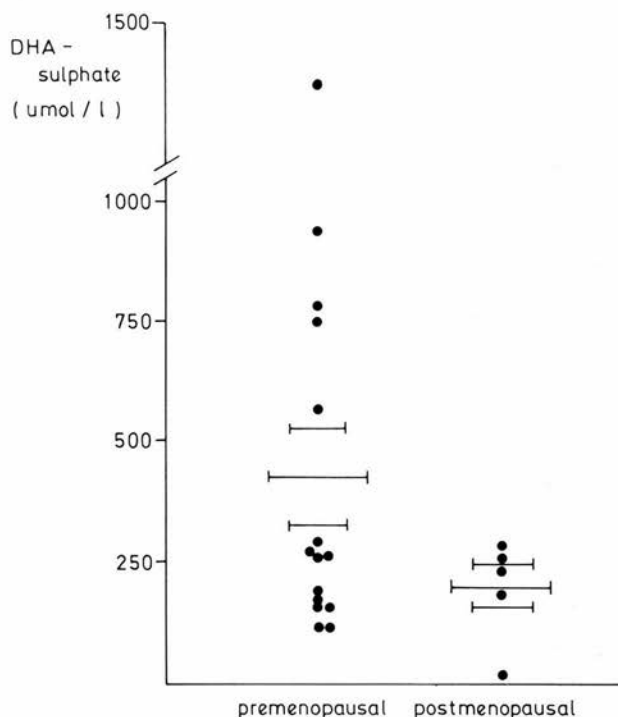


FIG. 3. Levels of DHA-sulfate in breast secretions from pre- and postmenopausal normal women. Lines are mean and standard error of the mean. Where more than one secretion was obtained from an individual, the values have been averaged.

from the same women. However, there was wide range in concentrations of DHA-sulfate in breast secretions (5-2,000 $\mu\text{mol/l}$). In addition, there was great variation in the secretion:plasma ratio of DHA-sulfate between different women. The data for the 21 normal women investigated are presented in Fig. 1 and show that DHA-sulfate concentrations in breast secretions always exceeded those in plasma, but the relative excess varied greatly, between seven- and 400-fold in different subjects. It was of interest to investigate parameters that might account for the differences in DHA-sulfate levels in breast secretions between different women; but before

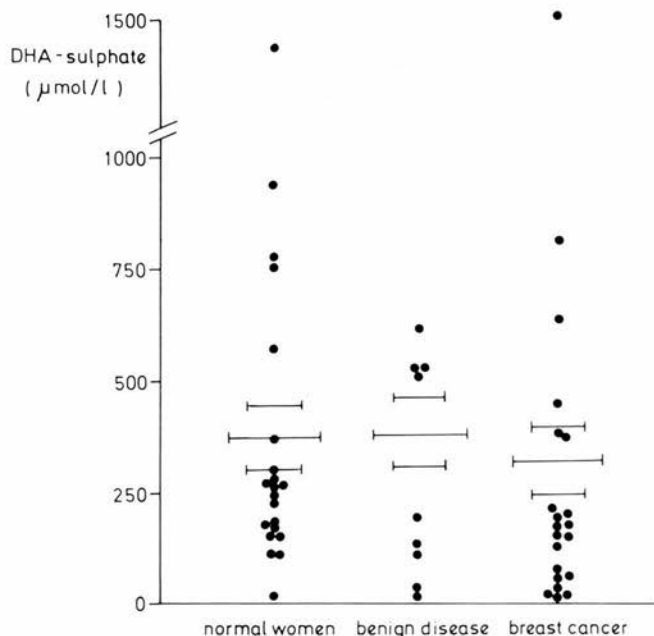


FIG. 4. DHA-sulfate concentrations in breast secretions from normal women, patients with benign breast disease, and those with breast cancer. Where more than one secretion was obtained from an individual, the values have been averaged.

doing so, it was necessary to determine the variation that might exist between secretions from the same individual.

We have already shown that concentrations of DHA-sulfate are of a similar magnitude in breast secretions from different ducts of the same breast and from different breasts of the same individual (4). To determine the consistency of DHA-sulfate levels in breast secretions with repeated sampling of individuals, secretions have been obtained, on more than one occasion, from the breasts of 4 premenopausal women. Results are presented in Fig. 2. Some variations in DHA-sulfate concentrations were apparent in secretions collected from the same breast on separate occasions, but the differences were small in comparison with those observed between different women. There was also no evidence for a decrease in DHA-sulfate concentration with continued sampling despite total volumes in excess of 1.5 ml being obtained from individual breasts. Thus, there is a relative uniformity of DHA-sulfate in secretions obtained from the breasts of individual normal women as studied over the short-term period.

Among other factors investigated to account for variation in DHA-sulfate concentration between breast secretions from different women were the effects of menopausal status and the presence of breast disease. No significant difference was apparent between DHA-sulfate levels in breast secretions from pre- and postmenopausal normal women (Fig. 3) although the mean value in the premenopausal

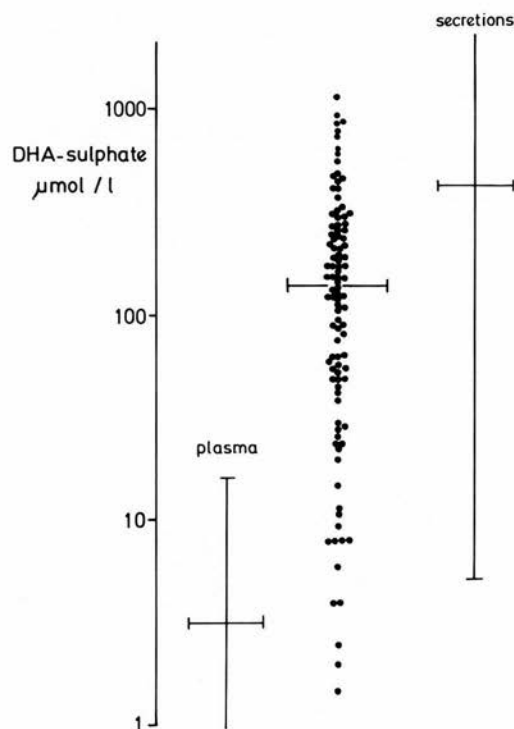


FIG. 5. Concentrations of DHA-sulphate in breast cyst fluids. Bars represent medians. Lines are range of values in breast secretions and plasmas from different women (included for comparison).

group was double that in the postmenopausal. However, the number of secretions studied in the postmenopausal group was small because of the relative difficulties in obtaining secretions from these women; it is possible that with larger numbers the difference observed may reach statistical significance.

Levels of DHA-sulfate in secretions from women with either benign or malignant breast disease are compared with those from normal women in Fig. 4. No significant differences were detected between the groups, but it is probably premature to exclude the possibility that disease affects the accumulation of DHA-sulfate in breast secretions. The groups of women investigated were not well matched; most normal subjects were premenopausal, whereas the majority of those with breast cancer were postmenopausal. Furthermore, in women with breast disease, no account has been taken of the breast in which the lesion was situated, and secretion may have been obtained from the unaffected breast. Studies of individuals comparing secretion from diseased and nondiseased breasts may provide more meaningful information.

Breast Cyst Fluids

All 100 cyst fluids analyzed contained DHA-sulfate as estimated by radioimmunoassay, but there was a wide range of concentrations (1.5–1,155 $\mu\text{mol} / \text{l}$). Like breast secretions, most cyst fluids contained high levels of DHA-sulfate, but ap-

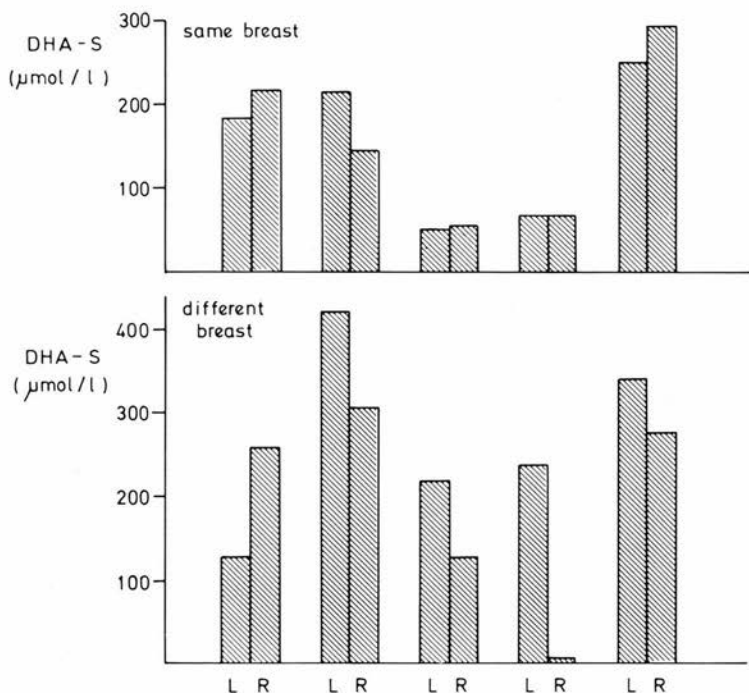


FIG. 6. DHA-sulfate concentrations in multiple cysts aspirated on the same occasion from the same breast (upper) and different breasts (lower) of the same individuals. L = left breast. R = right breast.

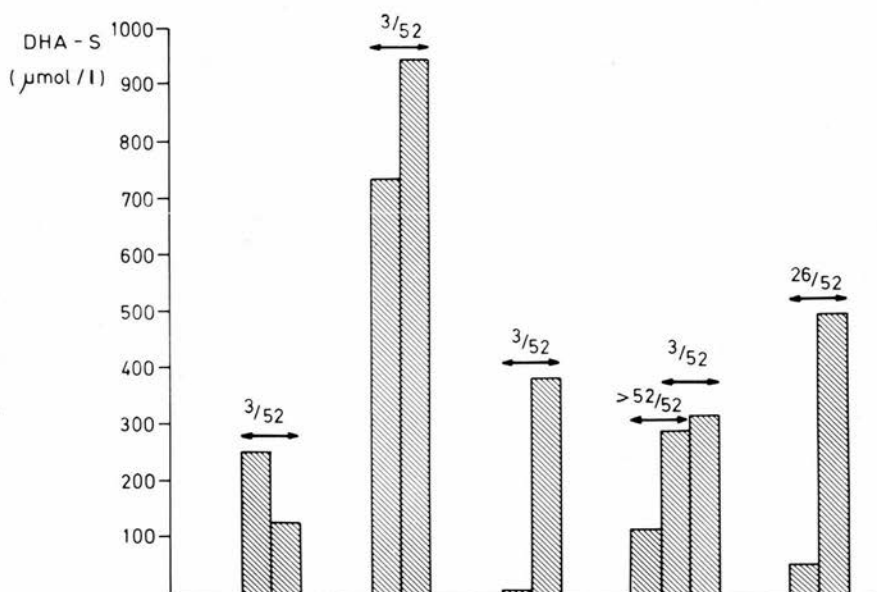


FIG. 7. DHA-sulfate concentrations in multiple cysts aspirated from the same breasts on separate occasions. The numbers within arrows represent the number of weeks elapsing between consecutive aspirations of the same breast.

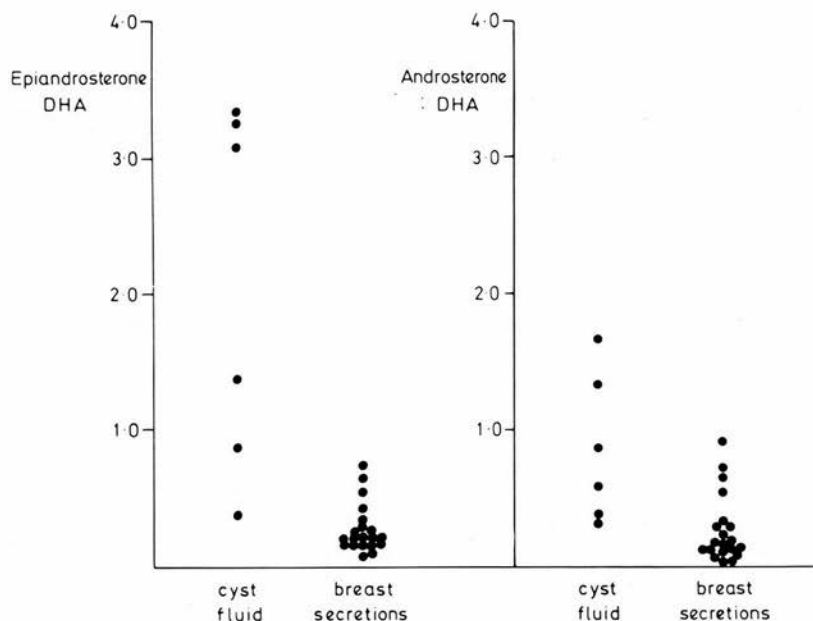


FIG. 8. Ratios of epiandrosterone:DHA and androsterone:DHA conjugates in breast cyst fluids and secretions.

proximately 15% contained values that were similar to those in plasma (Fig. 5). We have, therefore, examined factors that might account for the large variation in DHA-sulfate concentrations between different cysts. However, there was no correlation between DHA-sulfate levels and the age, menopausal status, parity, and, in premenopausal subjects, stage of the menstrual cycle of the women from whom the cysts were derived. Nor were DHA-sulfate levels related to the volume of cyst fluid aspirated.

Certain patients had multiple cysts and others had cysts aspirated on more than one occasion, and these have allowed us to assess variations within individuals. When multiple cysts were aspirated from the same breast on the same occasion, DHA-sulfate levels within the cysts often were comparable (Fig. 6). However, when cysts were aspirated from different breasts of the same individuals, larger variations in hormone concentration were observed (Fig. 6). Five women had cysts aspirated from the same breast on more than one occasion at intervals varying between 3 weeks and 2 years (Fig. 7). Large variations in DHA-sulfate levels were observed frequently in these cyst fluids although they were derived from the same breast. Such variations in DHA-sulfate between cysts fluids from individual women must complicate comparative studies between groups of different women.

Androsterone and Epiandrosterone Conjugates

Twenty breast secretions and 6 cyst fluids were analyzed by g.l.c.-mass spectrometry for DHA, androsterone, and epiandrosterone conjugates. Large amounts

of each were detected in all fluids. In breast secretions, DHA-sulfate always represented the major androgen conjugate (the ratios of androsterone and epiandrosterone:DHA were always <1 ; Fig. 8). By contrast, in cyst fluids, levels of epiandrosterone and, to a lesser extent, androsterone conjugates sometimes exceeded those of DHA (Fig. 8). The significance of the difference between breast secretions and cyst fluids and whether or not it represents metabolism within cysts remain to be elucidated.

SUMMARY

Both breast secretions from nonlactating women and breast cyst fluids contain large but variable amounts of DHA-sulfate and other closely related androgen conjugates. Little is known about how these hormones accumulate within breast fluids or what factors influence their concentration.

With regard to breast secretions, there appears to be a relative uniformity in DHA-sulfate levels within the breasts of normal women. Preliminary data have failed to detect significant differences in concentrations in secretions from patients with benign or malignant breast disease as compared with those from normal women.

Comparisons of DHA-sulfate levels in breast cyst fluids between different groups of women are hampered by the observation that multiple cysts in the same women, particularly if situated in different breasts or aspirated on separate occasions, can show widely differing concentrations.

Finally, the physiological significance of high concentrations of DHA-sulfate within the breast is unknown. The hormone has only weak biological activity, but it may be metabolized by the breast to form more active compounds (2).

ACKNOWLEDGMENTS

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Classification of human breast cysts according to electrolyte and androgen conjugate composition

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One hundred human breast cyst fluids have been analysed for sodium (Na^+), potassium (K^+) and dehydroepiandrosterone (DHA) sulphate. Concentrations varied greatly between individual cyst fluids, Na^+ from 20 to 185 mmol/l, K^+ from 5 to 160 mmol/l and DHA-sulphate from 1.5 to 87 $\mu\text{mol/l}$. Analysis of the inter-relationships between Na^+ , K^+ and DHA sulphate revealed two major sub-populations of cyst fluids—one group in which Na^+ levels were markedly in excess of K^+ and DHA sulphate concentrations were low and the other in which K^+ was the predominant cation and DHA sulphate concentrations were high.

Introduction

In Western countries, 7% of women present with a palpable cyst in the breast (Haagensen *et al.*, 1981). Women with cystic disease are believed to have an increased risk of developing breast cancer (for reviews see Azzopardi, 1979; Haagensen *et al.*, 1981), yet comparatively little is known about the aetiology of cysts or the composition and derivation of cyst fluid.

Recent work on the composition of cyst fluid has shown that concentrations of electrolytes (Gatzy *et al.*, 1979; Bradlow *et al.*, 1981b) and conjugates of androgens (Bradlow *et al.*, 1981a; Miller *et al.*, 1982) and oestrogens (Raju *et al.*, 1977) vary widely between individual cyst fluids.

The present study describes the interrelationships between concentrations of electrolytes and androgen conjugates and suggests a classification of cyst fluids on the basis of these constituents.

Materials and methods

One hundred breast cyst fluids were obtained by needle aspiration from 85 patients. In 75 subjects a single cyst was aspirated but, in 10, multiple cysts were drained (Table 1). Volumes varied from 0.7 to 50 ml.

*J. M. Dixon is a Wellcome Surgical Research Fellow.

TABLE 1. Electrolyte classification of multiple cysts

Patient	Breast 1		Breast 2	
	Cyst 1	Cyst 2	Cyst 3	Cyst 1
1	Na ⁺	Na ⁺		
2	Na ⁺	Na ⁺	Na ⁺	
3	Mix	Mix		
4	K ⁺	K ⁺		
5	K ⁺	K ⁺		
6	K ⁺	K ⁺	K ⁺	K ⁺
7	K ⁺			K ⁺
8	K ⁺			K ⁺
9	K ⁺	K ⁺	Mix	
10	Na ⁺	K ⁺		Na ⁺

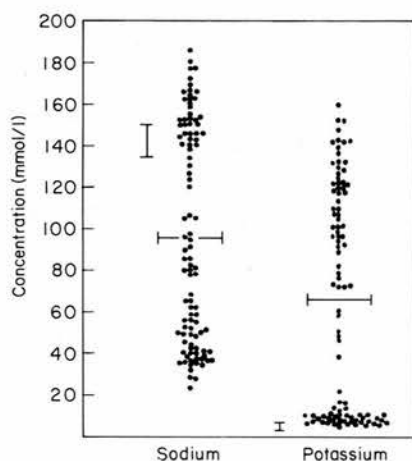


Figure 1. Concentrations of sodium and potassium in human breast cyst fluids. Horizontal lines represent mean level. Vertical lines represents reference range for human plasma.

Estimates of concentrations of Na⁺ and K⁺ were performed by flame photometry (EEL model 150 flame photometer) on cyst fluids diluted 1:200 in distilled water.

Androgen conjugates were measured by radioimmunoassay using an antibody described previously (Miller *et al.*, 1980). While this antibody is largely specific for dehydroepiandrosterone and its sulphate, it does cross-react with epiandrosterone conjugates. The results from the radioimmunoassay therefore reflect levels of both DHA sulphate and closely related androgen conjugates (levels of free DHA are very low in comparison with conjugated steroid). For simplicity, however, values from the radioimmunoassay have been expressed as units of 'DHA sulphate'. All statistical analyses were performed using non-parametric tests (Kendall Rank and Wilcoxon Rank tests as indicated).

Results

The levels of Na⁺ and K⁺ in the cyst fluids are shown in Figure 1. Values for both ions varied enormously and, in the case of potassium, were usually greatly in excess

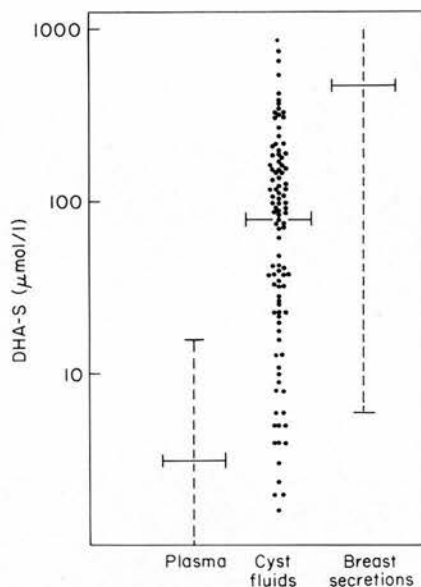


Figure 2. DHA-sulphate levels in human breast cyst fluids. Dotted vertical lines represent range in human plasma and breast secretions obtained by nipple aspiration. Horizontal lines represent median values.

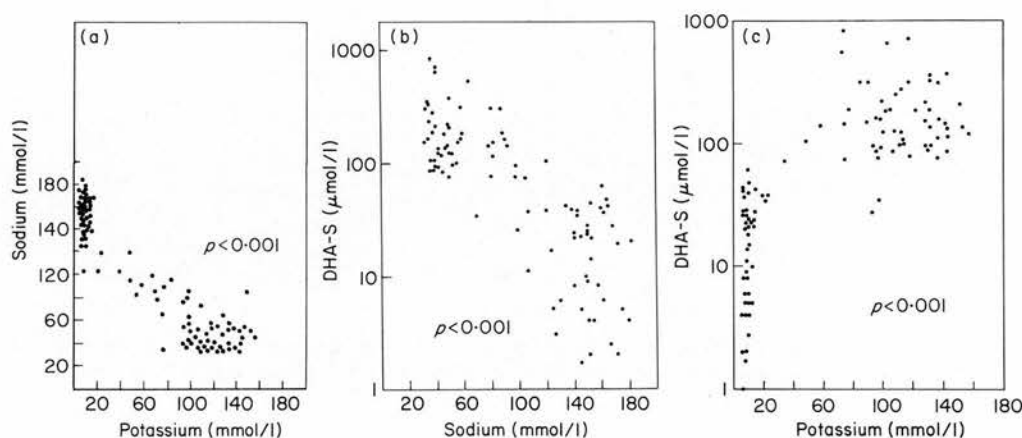


Figure 3. Relationships in human breast cyst fluid between (a) Na^+ and K^+ ; (b) DHA sulphate and Na^+ ; (c) DHA sulphate and K^+ . Significance values from Kendall rank test.

of the reference range in plasma. Distribution about the mean of values for both ions was suggestive of there being more than one population of cyst fluids.

The concentrations of DHA-sulphate in each of the 100 human breast cyst fluids is shown in Figure 2. Levels varied from 1.5 to 870 $\mu\text{mol/l}$ with a median value of 80 $\mu\text{mol/l}$.

Interrelationships between K^+ , Na^+ and DHA-sulphate are shown in Figures 3(a), (b) and (c). These were all statistically significant by the Kendall Rank test ($p < 0.001$ in each case). The correlations between Na^+ and both K^+ and DHA sulphate were

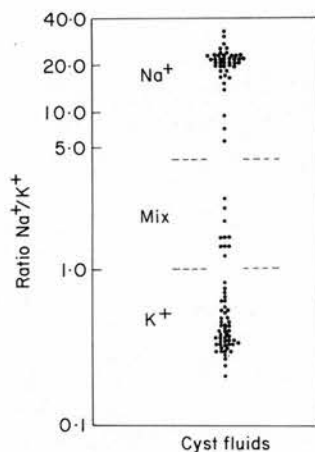


Figure 4. Ratio of Na^+/K^+ in human breast cyst fluids. Lines represent arbitrary divisions to give Na^+ (ratio > 4), Mix (ratio $1-4$), K^+ (ratio < 1).

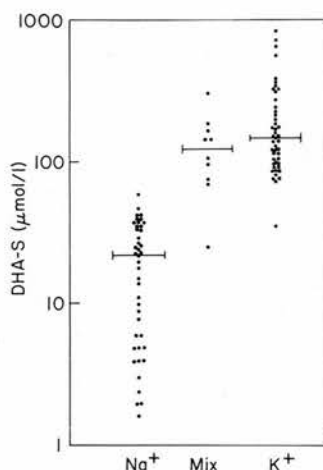


Figure 5. DHA sulphate levels in human breast cyst fluids subdivided according to electrolyte classification Na^+ represents fluids with $\text{Na}^+ : \text{K}^+ > 4$, mix fluids with $\text{Na}^+ : \text{K}^+ 1-4$ and K^+ fluids with $\text{Na}^+ : \text{K}^+ < 1.0$.

inverse relationships whereas that between K^+ and DHA sulphate was in a positive direction.

As the distribution of values for both Na^+ and K^+ suggested that there was more than one population of cyst fluids, the 100 samples examined were arbitrarily subdivided into three groups according to Na^+ and K^+ by means of their concentration ratio (Figure 4). One group was of 47 fluids in which the K^+ concentration exceeded that of Na^+ (K^+ fluids), another of 43 fluids in which the Na^+ concentration was above four-fold higher than that of K^+ (Na^+ cyst fluids), and a further group of 10 fluids with intermediate electrolyte values (mix cyst fluids). Values for DHA-sulphate in these subgroups of fluids are shown in Figure 5. Concentrations of DHA-sulphate in K^+ cyst fluids were significantly higher than the Na^+ group ($p < 0.0005$). Levels in the mix

category were similar to the K^+ group but significantly higher than the Na^+ group ($p < 0.001$).

Ten patients had multiple cysts aspirated on the same day. The electrolyte classification is shown on Table 1. In eight women, multiple cysts were of the same electrolyte group; two patients had cysts of different grouping although in one case the cysts co-existed within the same breast.

Discussion

Although the present study confirms that levels of Na^+ , K^+ and DHA-sulphate vary widely between different cyst fluids (Bradlow *et al.*, 1981*a, b*; Miller *et al.*, 1982), it has shown that there are significant associations between the concentration of these constituents. Thus, levels of Na^+ are inversely correlated with concentrations of both K^+ and DHA-sulphate. The values for Na^+ and K^+ are also not distributed symmetrically about their mean values and distribution is suggestive of more than one population of cyst fluids. It is possible to use the relative concentrations of Na^+ and K^+ to identify presumptive sub-groups of cyst fluids. This arbitrary classification based on electrolyte composition also subdivides the cyst fluids into those with high or low levels of DHA sulphate.

It therefore can be shown that 90% of cyst fluids fall into one of two major sub-groups—one type (K^+ fluids) contains high concentrations of K^+ and DHA-sulphate and low concentrations of Na^+ whereas the other type (Na^+ fluids) has the reverse composition. Bradlow and his colleagues (1981*b*) have independently classified cysts on electrolyte composition but found a higher proportion of K^+ cysts as compared with the present study.

The derivation of Na^+ and K^+ subgroups of cyst fluids still remains to be defined. They may reflect different sources of constituents or differences in secretory activity of the epithelium lining the cysts. The composition of Na^+ cysts is akin to that of plasma in respect of Na^+ , K^+ and DHA-sulphate. However, although DHA-sulphate levels in K^+ cyst fluids are similar to those in breast secretions obtained by nipple aspiration (Miller *et al.*, 1981), Na^+ is the predominant ion in such breast secretions. Simple accumulation of these breast secretions is therefore unlikely to account for the formation of K^+ cyst fluids.

It seems more likely that the constituents of K^+ cyst fluids result from apocrine activity by the epithelium lining the cyst. Apocrine secretions characteristically contain high concentrations of K^+ and DHA-sulphate (Labows *et al.*, 1979). Our preliminary results examining cells lining the walls of excised cysts would support this concept.

The relevance of this classification of breast cyst fluids still remains to be elucidated and it should be noted that different types of cyst fluids may co-exist within the same breast although it is more usual for multiple cysts to be of the same type. It may be, however, that a sub-population of cysts occurs in women at high risk of epithelial proliferative changes including cancer.

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The morphological basis of human breast cyst populations

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Forty human cysts have been examined to determine the relationship between the epithelial lining and the content of sodium (Na^+) and potassium (K^+) in the cyst fluid. The ratios of Na^+ to K^+ for cysts lined by flattened epithelium were higher in all cases than the values obtained for cysts lined by apocrine epithelium. These findings suggest a morphological basis for the two populations of human breast cyst fluids which can be defined on cationic content.

Human breast cyst fluids can be separated into two major populations on the basis of the relative concentrations of sodium (Na^+) and potassium (K^+) (1, 2). The two major groups are: (i) fluids in which concentrations of K^+ exceed those of Na^+ ; and (ii) fluids in which levels of Na^+ greatly exceed those of K^+ . Cyst fluids in which K^+ is the predominant cation contain high concentrations of the androgen conjugate dehydroepiandrosterone sulphate (DHA sulphate) (2) and contain the secretory (11s) form of immunoglobulin A (IgA) (3, 4), indicating that the epithelium lining such cysts is active and secretory. In contrast cyst fluids in which Na^+ predominates, contain low levels of DHA sulphate (2) and the non secretory (7s) form of IgA and would be indicative of a less active epithelium. To date, however, no evidence has been reported to show that differences in breast cyst composition are associated with the nature of the lining epithelium. The aim of the present study was to investigate the relationship between the morphology of epithelium of human breast cysts and the cationic content of cyst fluid.

Materials and methods

Morphological assessment of the epithelium lining 40 human breast cysts was performed either by: (i) centrifuging an aliquot of cyst fluid and examining the stained deposit cytologically (26 cysts from 22 patients); or (ii) dissecting cysts from tissue obtained at mastectomy or biopsy and submitting the cyst wall for histological processing and serial sectioning (7 cysts from 6 mastectomy specimens and 7 cysts from 6 biopsy specimens).

The aspirates of cyst fluid were stained by Papanicolaou's technique (Pap) and by periodic acid Schiff after diastase digestion (PAS diastase). Dissected cyst specimens were stained by haematoxylin and eosin (H & E) and PAS diastase. PAS diastase positive granules in the cytoplasm of cells were used as a positive method of identification of apocrine epithelium (5). All cyst fluid aspirates and dissection specimens were assessed by a consultant pathologist with a specific training in breast histology and cytology.

The concentration of Na^+ and K^+ in the 40 cyst fluids was measured by flame photometry (EEL model 150 flame photometer) on a 1 in 200 and a 1 in 400 dilution of cysts fluid in distilled water.

Results

It was possible to identify two major types of epithelial cells in both cytological and dissection preparations: (i) acidophilic cells (on H & E) with copious granular cytoplasm containing PAS diastase positive granules, luminal apical snouts and nuclei showing prominent nucleoli—*apocrine* epithelium; (ii) basophilic cells (on H & E) having less cytoplasm and containing no PAS diastase positive granules—*flattened* epithelium.

Figs. 1 and 2 show examples of apocrine and flattened epithelial cells in breast cyst aspirates. Fig. 3 shows the specific glycolipid granules in the cytoplasm of apocrine cells. Figs. 4 and 5 demonstrate apocrine and flattened epithelium in dissection specimens with Fig. 6 showing the apical position of the glycolipid granules in apocrine epithelium.

In this series no cyst showed a mixture of apocrine and flattened epithelium in either aspiration or dissection specimens. In 22 cysts the lining was assessed as apocrine (14 aspiration and 8 by dissection) and in 18 the lining was

flattened simple epithelium (12 by aspiration and 6 by dissection).

The correlation between the morphology of the lining epithelium as assessed in cytological preparations and dissection specimens and the Na^+/K^+ ratio is shown in Fig. 7. Cysts with flattened epithelium had significantly higher Na^+/K^+ ratios than cysts lined by apocrine epithelium ($P < 0.001$ Wilcoxon's Rank Sum test). On the basis of Na^+/K^+ ratios it was possible

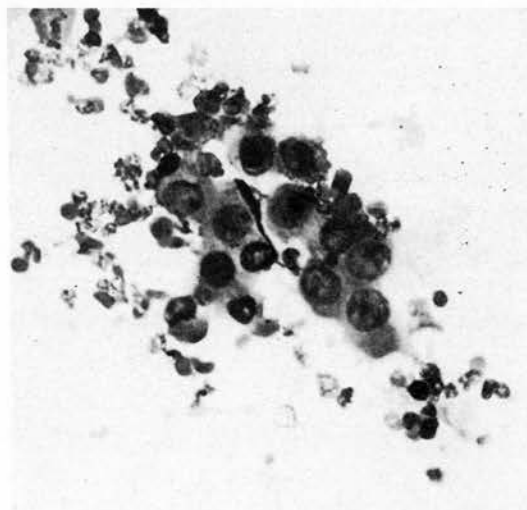


Fig. 1. Cytological preparation of a cyst aspirate showing apocrine cells. The cells have copious pale granular cytoplasm with vesicular nuclei showing prominent nucleoli (Pap $\times 480$).

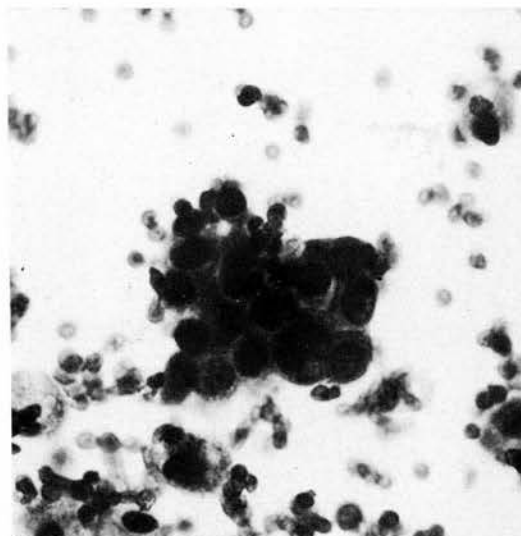


Fig. 2. Cytological preparation of a cyst aspirate showing simple flattened epithelium. The cells have small amounts of cytoplasm, deeply staining nuclei and no special features (Pap $\times 480$).

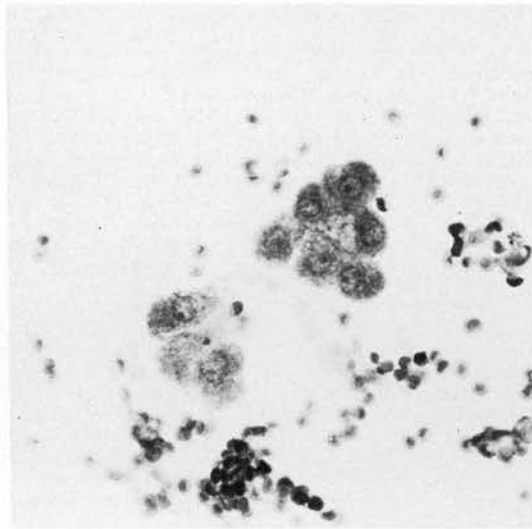


Fig. 3. Cytological preparation of a cyst aspirate stained to show the specific glycolipid granules found in apocrine cells. The vesicular nuclei with prominent nucleoli, also a feature of apocrine epithelium, are easily seen (PAS diastase $\times 480$).

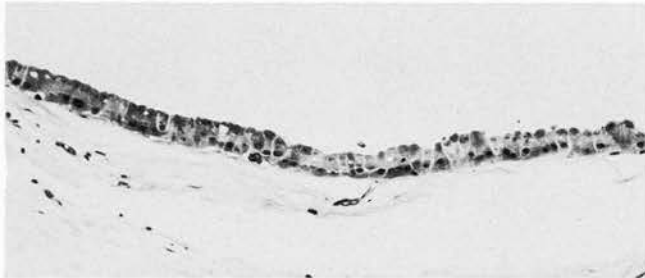


Fig. 4. Dissection specimen of an apocrine cyst. The cells are columnar and have basally situated nuclei, abundant cytoplasm and apical snouts containing deeply staining intracytoplasmic glycolipid granules (H & E $\times 128$).



Fig. 5. Dissection specimen of a flattened epithelial cyst. The epithelium is attenuated and flattened and few cells are visible in any one section (H & E $\times 256$).

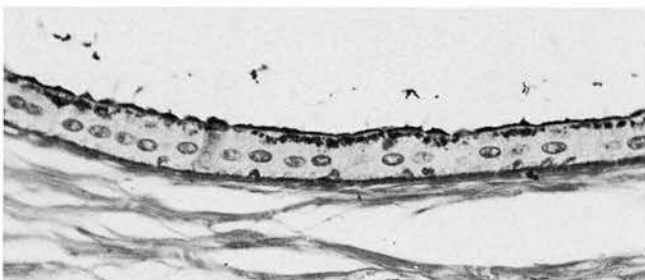


Fig. 6. Dissection specimen showing the apical position of the glycolipid granules in apocrine epithelium (PAS diastase $\times 256$).

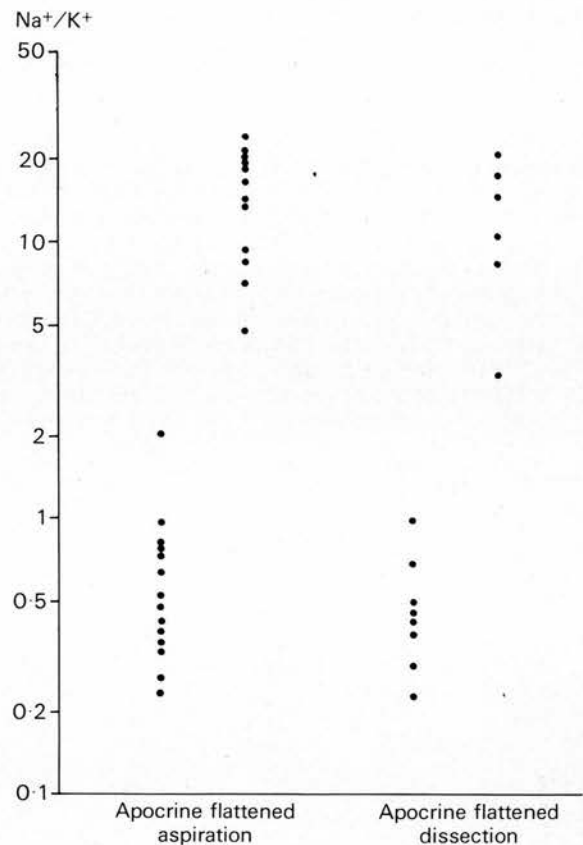


Fig. 7. The ratios of sodium (Na^+) and potassium (K^+) in the cysts where the epithelium was assessed as apocrine or flattened by examination of aspiration and dissection preparations.

to distinguish completely between apocrine and flattened epithelial cysts, those cysts lined by apocrine epithelium having a ratio of 2 or less and those lined by simple flattened epithelium a ratio of greater than 3.

Discussion

Approximately 7 per cent of all women in the Western world develop a breast cyst (6). Certain studies have shown that these women are at increased risk of developing breast cancer (5, 6) although others have been unable to confirm this finding (7). In view of their frequency and their possible relationship to breast cancer, it is surprising that little is known of the composition or derivation of human breast cysts. Recently breast cyst fluids have been divided into two major groups on the basis of the relative concentrations of Na^+ and K^+ (1, 2, 8). These two groups of fluids have also been shown to contain significantly different levels of the androgen conjugate DHA sulphate and to contain different molecular forms of IgA (2-4). The present study has demonstrated that the epithelia lining these two groups of cysts are morphologically different. All cysts where the Na^+/K^+ ratio was 2 or less were lined by apocrine epithelium and those with a ratio of greater than 3 were lined by flattened epithelium.

This subdivision on the basis of the Na^+/K^+ ratio is similar to that used by us to discriminate between cysts with high and low DHA sulphate (1, 2). Cysts with a low Na^+/K^+ ratio had high levels of DHA sulphate. Such cysts have been shown in the present study to be lined by apocrine epithelium. It is of note that apocrine secretion from axillary skin has also been shown to contain high concentrations of DHA sulphate (9).

Electron microscopic studies of breast cyst epithelium have shown that apocrine cells contain many mitochondria and apical secretory granules, in contrast to flattened epithelial cells which contain few organelles (10). Apocrine secretion is performed by expelling the contents of the intracellular secretory granules and is more accurately termed merocrine (5). The

finding of high potassium, the major intracellular cation, in cysts lined by apocrine epithelium, correlates well with what is known of the ultrastructure and method of secretion of epithelium of apocrine type.

Studies of patients with multiple cysts in one or both breasts have shown that, in the majority, the cysts had a similar electrolyte composition (2) and were therefore likely to be lined by the same type of epithelium. This observation suggests there may be a factor which determines whether patients develop cysts lined by apocrine or flattened epithelium. Apocrine change has been reported to occur more frequently in populations at high risk of breast cancer (11–13) and the results from this study show that the presence of apocrine epithelium in cystic disease may be detected by electrolyte analysis of breast cyst fluid. It is therefore possible that such analysis will be helpful in identifying patients at increased risk of breast cancer.

Acknowledgements

Thanks are accorded to Dr T. J. Anderson and Dr J. Lamb of the University Department of Pathology for assessing all cytological and histological preparations and Miss C. Hughes for secretarial assistance. Dr J. M. Dixon is a Wellcome Surgical Research Fellow and acknowledges the support of the Wellcome Trust.

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Breast News



NUMBER
ONE

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palpably effective

The success of Danol in benign breast disease has been demonstrated in numerous open studies. Now confirmation has come from a recent placebo-controlled trial.

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WINTHROP

The economics of benign breast disease

By Michael Baum, Hon Director, CRC Clinical Trials Centre, and Hilary Cooper, Remploy Research Nurse, Kings College Hospital Medical School, London.

This paper attempts to estimate the cost of benign breast disease, both from the point of view of the patient and that of the health service, and makes recommendations as to how these costs may be reduced.

During the first two years of its existence the breast clinic at King's College Hospital (established in January 1980) diagnosed 1272 patients. Of these, 1028 had some form of benign breast disease or no apparent abnormality and 244 had cancer, the single most common diagnosis. Table 1 shows the numbers of each diagnostic sub-group.

Just under half the patients who

attended the clinic were discharged following clinical examination supplemented by mammography in selected cases. Patients presenting with simple cysts (13%) were dealt with by single or multiple needle aspiration (see below); 19% had discrete solid lumps or areas of mammographic suspicion which were subjected to open biopsy when no malignancy was found. This number is identical with

the number of patients whose biopsy ultimately confirmed the presence of cancer. The proportion of one benign biopsy to one malignant biopsy compares favourably with others reported from diagnostic clinics such as this one^{1,2}. A higher benign to malignant biopsy proportion might be expected from screening clinics³.

The patient

Any symptom related to the breast causes the patient intense anxiety, mainly due to fear of cancer. Just before attending the clinic, this anxiety reaches a peak. It can be detected by asking the women about insomnia and the ingestion of alcohol or anxiolytics in the week preceeding her first appointment at the clinic. If a biopsy is indicated, a further intense period of anxiety builds up beforehand and while waiting for the result. Any attempts to reduce the biopsy rate, therefore, must be applauded.

In addition, serious cosmetic problems may result from repeated small biopsies, or removal of breast quadrants in the search for small mammographic abnormalities.

There are also small numbers of women who are developing a neurosis because of the publicity given to breast self-examination and early diagnosis of cancer. They repeatedly attend the clinic for reassurance.

While it has taken many years to educate general practitioners to view all discrete lumps in the breast with suspicion, rapidly referring patients to the surgeon, we think that general practitioners now act cautiously and need to be re-educated. This was discussed in detail in a recent publication⁴. Table 2 lists our opinion on which patients general practitioners should refer to the clinic.

Once the general practitioner is convinced that a specialist opinion is required, the waiting period must be reduced to a minimum. If it is impossible for an open-house policy to be offered in a hospital, then the breast clinic should at least ensure that no

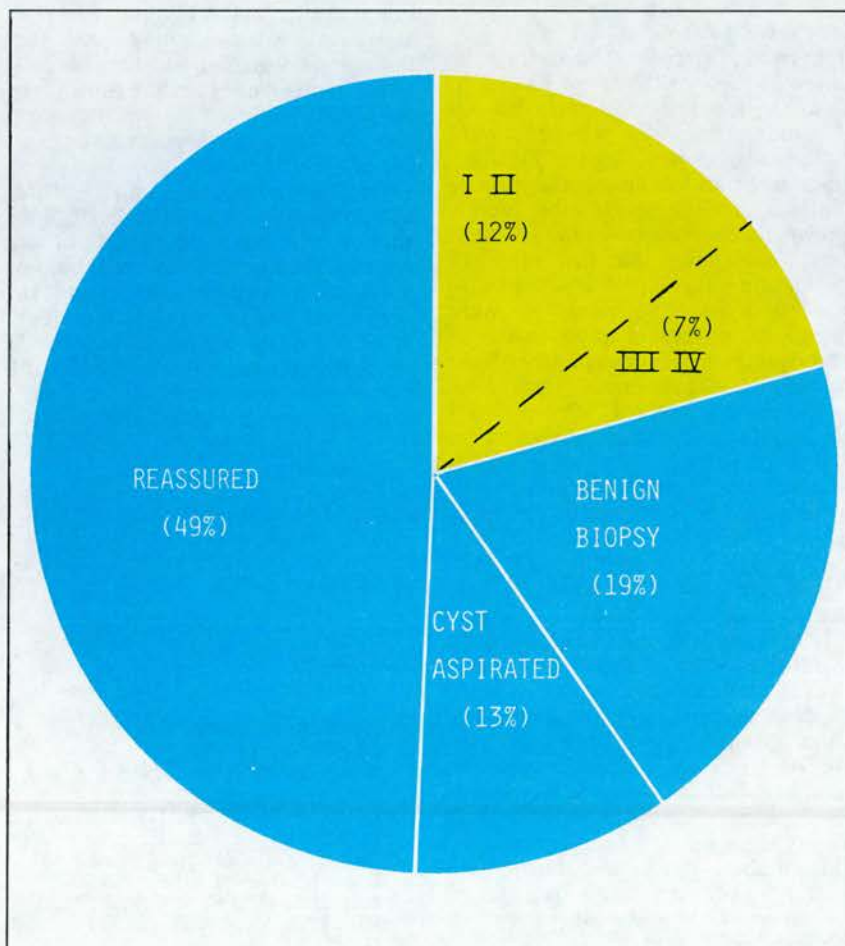


Table 1. KCH Breast Clinic (Jan. 1980-Dec. 1982)

Diagnosis	No.
Cancer	244
'Lumpy breasts'	224
Normal breasts	196
Cysts	167
Mastalgia	142
Fibroadenoma	116
Duct ectasia complex	63
Epitheliosis	53
Sclerosing adenosis	
Duct papilloma	19
Miscellaneous	48
Total	1272

woman has to wait more than a week following the receipt of a referral letter. Once the patient has been seen at the clinic, if, following clinical examination and mammography, rapid reassurance cannot be provided, then histological diagnosis of the suspicious area must be expedited. Cyst aspiration or aspiration cytology should become more widely available and out-patient needle biopsy, ideally with frozen section, may also reduce this period of uncertainty. Finally, we think it is time to evaluate critically the whole subject of breast self-examination and to consider whether or not it saves lives⁵.

The Health Service

It costs the NHS a great deal of money to set up a breast clinic. The clinic takes up the time of medical, nursing and clerical staff and its mammography service involves capital outlay for equipment and sessional payments for radiographers and radiologists. Inevitably, too, the establishment of such a clinic increases the demand for beds and theatre time for in-patient biopsy.

Hospital costs would be reduced if general practitioners shouldered more responsibility for women presenting with breast symptoms. Also, out-patient biopsy or cytology would reduce the need for theatre time and bed occupancy, and a policy of repeatedly aspirating cysts might reduce the biopsy rate.

Surprisingly, many clinicians believe that cyst aspiration is hazardous because an intracystic cancer might be missed or a solid tumour, if punctured, might disseminate the cancer. However, a number of recent publications

Table 3. Outcome of biopsies

Indications	No.	Outcome	
		Benign	Malignant
Solid lump	1	0	1
Bloody tap	2	0	2
Suspicious mammograms	9	9	0
Residual lump	26	26	0
Recurrent cyst			
Total	38	35	3

have demonstrated the safety of the procedure^{6,7}, and, with this in mind, we intend to critically review our own policy.

All women presenting at our clinic with solitary cysts are treated by needle aspiration. On each reattendance, a new cyst appearing in a different part of the same breast, or in the other breast, is aspirated. Biopsy is indicated only if any of the lumps turn out to be solid, if a residual lump is found at the site of aspiration at one month follow-up, if frank blood is found in the aspirate or if recurrent cysts occur at the same site as the original aspirated cysts. Finally, biopsy is, of course, indicated if mammography is reported as suspicious.

In the two-year period under review, 130 cases were managed in this way. Seventy-one per cent of patients were dealt with at a single aspiration, 20 per cent at two aspirations and the remainder required five or more aspirations, many of these patients continuing to return with new cysts at each visit. Of the total group, 29 per cent were submitted to open biopsy. The outcome is shown in Table 3. Of these 38 patients, only three were subsequently shown to have cancer. One of these was a solid lump that had appeared during the follow-up of a woman with multiple cysts. The other two were intracystic cancers demonstrated by bloody fluid in the cyst aspirate. All the suspicious mammograms were false positive and not one of the cases with a residual lump or recurrent cyst at the site of the first aspiration was positive. A policy of cyst aspiration seems safe. Mammograms performed following cyst aspiration were not only unhelpful but positively misleading and solid lumps and bloody taps must be subjected to excision biopsy.

There appear to be two distinct cyst syndromes. One group of women presented with a solitary cyst which could be dealt with in one or two visits. The other group (about 10 per cent) continued to form cysts. To date, an analysis of all demographic factors and drug history has failed to distinguish the type of women likely to develop one of these syndromes. Further research into the endocrine background of these two groups of patients is obviously indicated.

Our policy for mammography has now changed. All patients over the age of 30 referred to the clinic with breast symptoms now have their mamogram performed before the clinical examination, as we suspect that a recently aspirated cyst may cause the radiologist difficulty in interpretation.

Conclusions

Benign breast disease is a common problem which creates severe anxiety in many women and a heavy burden on an overstretched National Health Service. A policy of re-educating general practitioners when it is safe not to refer, may reduce this problem. Cyst aspiration or needle aspiration cytology may also reduce the burden to both patient and Health Service. A critical evaluation of the cost effectiveness of mammography and breast self-examination is also urgently required. In so far as patients are presenting when the tumours are smaller and there is less node involvement than previously experienced, self-examination may save lives but it may also simply prolong the period of observation.

Finally, we think it is important for all specialists to recognise their ignorance in the area of benign breast disease. We do not know its aetiology or natural history, therefore it must be very difficult to evaluate its treatment! A great deal more research is needed, yet research costs money and most of the money available is directed into studies concerning breast cancer.

A complete version of this paper will shortly be published as part of the symposium proceedings of the Journal of the Royal Society of Medicine.

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Table 2. When referral is indicated

Refer	Do not refer
Discrete lump	Vague lumpiness (particularly if occurring just before menses)
Recent nipple retraction	Tender and/or enlarged costochondral junction
Circumferential nipple retraction	Long-standing nipple retraction
Serous/bloody nipple discharge	Linear nipple retraction, white/cream/green nipple discharge
All ulcers	Superficial thrombophlebitis
Eczema	Intertrigo
Mastalgia starting after 30 years of age (particularly if unilateral or non-cyclical)	Bilateral cyclical mastalgia under 30 years of age.

Editorial comment

by Michael Baum

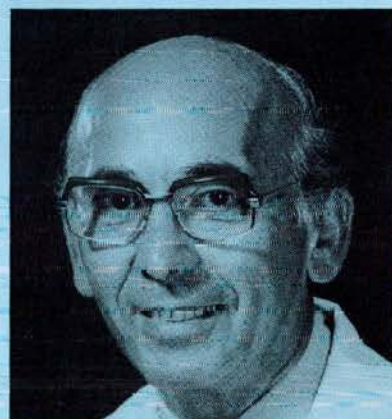
“When I was first approached by Winthrop Laboratories and the Medical News Group to edit a new journal relating to breast disease my initial response was one of dismay and rejection of the offer on three counts. My first retort was ‘Not another journal! My desk is already cluttered with unread back numbers of serious scientific journals as well as many of the giveaway medical journals. Secondly, I suggested in a somewhat sanctimonious way that I would rather not have my name associated with anything that was unequivocally promotional in nature, and, finally, (always remember the real reason is given last) I was far too busy to take on yet more responsibility with the inevitable persecution of meeting deadline after deadline. Nevertheless, I saw no harm in prolonging the discussions for a little while in the amiable company of my putative co-editors over an excellent dinner. Somewhat mellowed by the fine food and wines, we began to see how my initial reactions could be neatly turned upon themselves and used to their own advantage. Firstly, we all agreed there are far too many journals and it is impossible these days to keep up-to-date with the literature. Say, if someone were to do this for us and produce a service booklet with abstracts of the literature within our field of interest and regular up-to-date reviews by experts in fields co-terminus to these interests?

Secondly, there surely can be nothing sinister in a journal which is unashamedly promotional, as the aims of industry and the aims of academic progress can often be served by the same mechanism if these exchanges are conducted in an openhanded manner. After all there is excellent precedent for the type of journal that we are launching and I think it is fair to acknowledge at this point the welcome contributions made by such journals as *Cancer Topics* (sponsored by Lederle) and *Reviews on Endocrine Related Cancers* (sponsored by ICI).

Finally, as far as my last objection was concerned, the publishers reassured me that they would have everyone else to do the work and I would merely have to sit back and take the credit. So at last, after the gestation period of about a year, ‘*Breast News*’ emerges from the womb as an infant prodigy. Because of the commercial interests of the sponsors the emphasis of this journal will be directed at ‘benign breast disease’ and in fact that was the first title we thought of for this journal. However, we soon realised that this was far too narrow an objective to take, as the demarcation between benign and malignant disease is often unclear and to some extent artificial and one of the important aspects of a study of benign disease is the mechanism by which this may or

may not express itself subsequently as invasive carcinoma. Nevertheless, it is still intended to concentrate on benign breast disease as it is under-researched and the impact it makes on the lives of women throughout this country under-estimated. For example, the first article in the first issue of this journal, which relates to the experience of one busy breast clinic, emphasises the predominance of benign breast disease amongst women presenting with symptomatic breasts and the cost to the patient in terms of misery and anxiety, together with the cost to the Health Service in terms of clinical resources.

We hope this journal will be of value and interest to busy clinicians throughout the United Kingdom. We hope that occasionally we will produce controversy and we would, therefore, encourage a lively correspondence column. We hope that the abstract service and diary will be of practical help and that the review articles will also keep you up to date in areas outside your own immediate specialty. You may, of course, choose to throw the wretched thing away, but we intend that the journal will have shelf-life and for this reason rigid binders will be provided within which to store your copies. We would like to think that this infant prodigy will thrive and as my brother (the paediatrician) is wont to say, ‘Breast fed is best fed’ and I sincerely hope that you will find that ‘*Breast News*’ is good news.”



The Editorial Board: (Left) Professor Michael Baum, King's College Hospital Medical School, London. Mr Paul Preece, Ninewells Hospital and Medical School, Dundee. (Right) Dr Basil Stoll, St Thomas' Hospital, London.

Human breast cystic disease

J. M. Dixon, Wellcome Surgical Research Fellow, and W. R. Miller, Lecturer, University Department of Clinical Surgery, Royal Infirmary, Edinburgh, EH3 9YW.

INTRODUCTION

Breast cysts were first distinguished from carcinomas in 1829 by Astley Cooper¹. They are the commonest cause of a benign breast mass affecting approximately 7 per cent of all women in the Western World². It has been reported that these women may be at increased risk of subsequent breast cancer^{2,3}. In view of the length of time which has passed since their identification, their frequency and association with hyperplasia and breast cancer, it is surprising that comparatively little is known of the content and derivation of human breast cysts.

Composition

Recently electrolytes^{4,5} and hormones^{6,7} in cyst fluids have been measured and shown to vary widely between individual cysts. We have measured sodium (Na^+), potassium (K^+) and the androgen conjugate dehydroepiandrosterone sulphate (DHA sulphate) in 100 cyst fluids and confirmed this wide variation. (Figures 1 and 2). However, we found significant interrelationships between these three substances, with inverse correlations between Na^+ and both K^+ and DHA sulphate and a direct correlation between K^+ and DHA sulphate (Figures 3a, b and c). It was also evident that values for Na^+ and K^+ were not

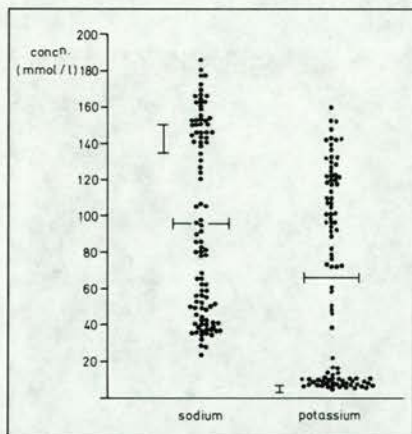


Figure 1. Concentrations of sodium and potassium in human breast cyst fluids. Horizontal lines represent mean values. Vertical lines represent reference range for plasma.

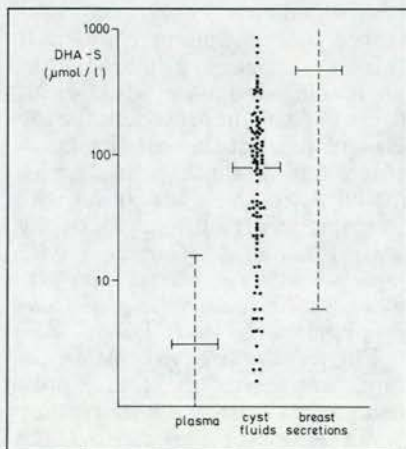


Figure 2. DHA sulphate levels in human breast cyst fluids. Dotted vertical lines represent range in human plasma and breast secretions obtained by nipple aspiration. Horizontal lines represent median values.

normally distributed around the mean, indicating that there may be more than one population of cyst fluids. This was particularly evident in a plot of the ratio of Na^+ to K^+ (Figure 4). Cyst fluids could be divided into two groups, one having a high and the other a low Na^+/K^+ ratio. DHA sulphate concentrations in these two populations were significantly different, levels being higher in fluids with low Na^+/K^+ ratios (Figure 5).

Further studies have shown that pH, concentration of albumin and IgG and type of IgA also vary in the two populations of cyst fluids as defined by electrolyte content.

Relationship of epithelial lining of cysts and cyst fluid composition

Morphological studies of the epithelium lining breast cysts have been undertaken to determine whether differences in the composition of the two populations of breast cysts are related to the nature of the lining epithelium. The epithelium lining 40 breast cysts was characterised by either histology or cytology. This was then compared with the composition of the cyst fluid.

Two types of epithelium were identified in both histological and cytological preparations:-

- (1) acidophilic cells on haematoxylin and eosin stained sections (H &

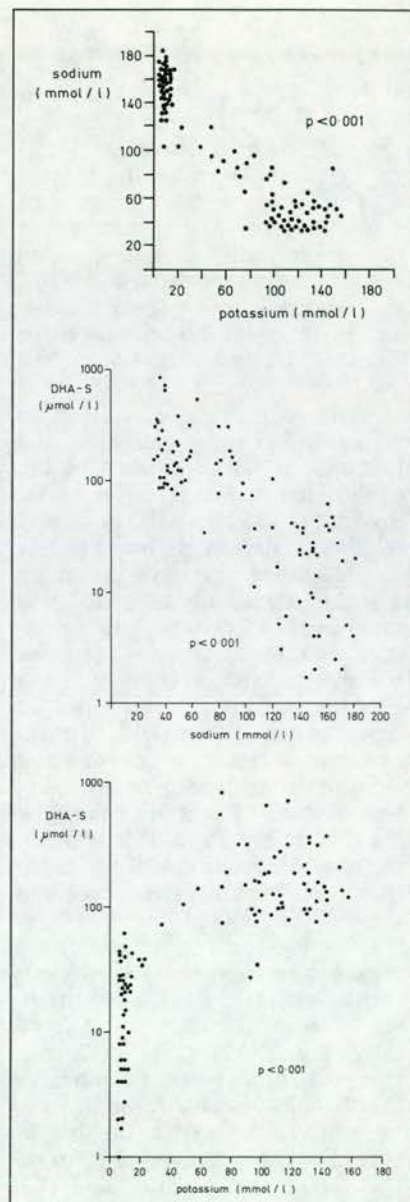


Figure 3. Relationships in human breast cyst fluid between (a) Na^+ and K^+ (b) DHA sulphate and Na^+ (c) DHA sulphate and K^+ . Significance values from Kendal rank test.

E), with copious cytoplasm containing granules which stain by the periodic acid Schiff (PAS) technique after diastase digestion, have luminal snouts and nuclei with prominent nucleoli — APOCRINE epithelium.

- (2) basophilic cells (on H and E) having less cytoplasm with no specific features — FLATTENED epithelium.

Figures 6, 7, 9 and 10 show examples of apocrine and flattened cells in histological and cytological preparations with the PAS positive glycolipid granules characteristic of apocrine epithelium being demonstrated in Figures 8 and 11.

Comparison of the epithelial lining of the cyst as assessed in histological

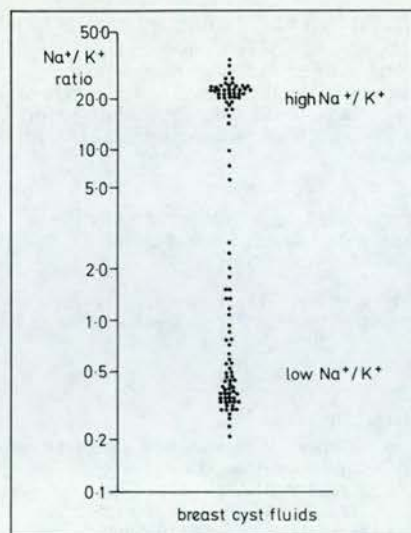


Figure 4. Ratio of Na^+/K^+ in human breast cyst fluids. Two populations are evident, one with a high and the other with a low Na^+/K^+ ratio.

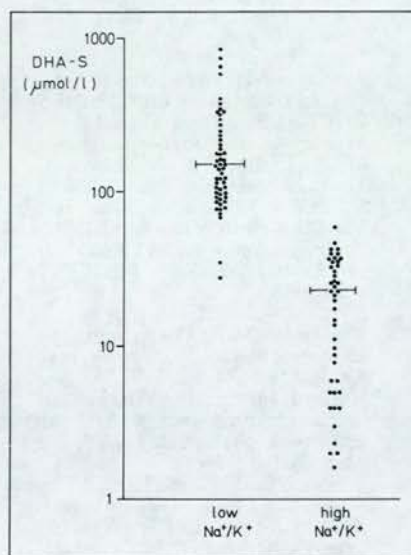


Figure 5. DHA sulphate in human breast cyst fluids subdivided according to electrolyte classification into fluids with high Na^+/K^+ and low Na^+/K^+ ratio.

and aspiration specimens and the Na^+/K^+ ratio in cyst fluid (Figure 6) showed all cysts lined by apocrine epithelium had a lower Na^+/K^+ ratio than those lined by flattened epithelium. Similarly subdivision of cysts according to histology of the epithelial lining split DHA-sulphate values into two separate groups, those with apocrine epithelium having high concentrations and those with flattened epithelium having low concentrations of DHA sulphate (Figure 13).

Apocrine cyst fluids thus have a low Na^+/K^+ ratio and high concentrations of DHA sulphate. Apocrine secretion occurs by expulsion of cellular contents via intracytoplasmic vacuoles² and the finding of high potassium concentrations in fluids derived from apocrine

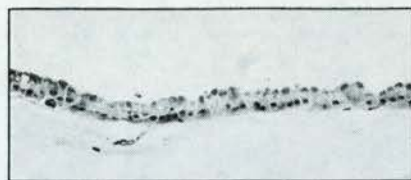


Figure 6. Histological specimen of an apocrine cyst. The cells are columnar, have basally situated nuclei, apical snouts and abundant cytoplasm containing intracytoplasmic glycolipid granules (H & E $\times 240$).



Figure 7. Histological specimen of a flattened epithelial cyst. The epithelium is flat and attenuated and has no specific features (H & E $\times 480$).

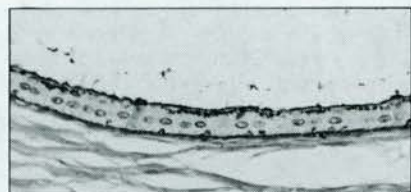


Figure 8. Histological specimen showing the apical position of the PAS positive glycolipid granules in apocrine epithelium. (PAS Diastase $\times 320$).

cells is consistent with this activity. It is of interest that apocrine secretion from axillary skin has been shown to contain high concentrations of DHA sulphate⁸. It is also of note that the colour range of apocrine axillary sweat (yellow to green to bluish black) is identical to that seen in breast cysts⁹.

Relationship of cyst fluid populations to natural history and breast cancer risk

Whilst two populations of breast cysts may be defined on the basis of composition and the nature of epithelial lining, it remains to determine whether these two subgroups have any clinical relevance. Preliminary studies suggest that these two groups differ in terms of their natural history and their association with the subsequent development of breast cancer.

We know that about half of all women, who present with cystic disease will have a single cyst, a third of patients will develop between 2 and 5 cysts and the remainder have in excess of 5². Our own data from a prospective analysis of 100 consecutive patients followed over 2 years show 45 per cent of patients had 1 cyst, 46 per cent had 2-5 cysts and 9 per cent had in excess of 5. All cysts aspirated from these 100 patients were classified on the basis of electrolyte composition as apocrine ($\text{Na}^+/\text{K}^+ < 3$) or flattened ($\text{Na}^+/\text{K}^+ \geq 3$).

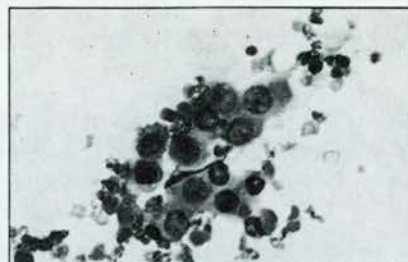


Figure 9. Cytological preparation of a cyst aspirate showing apocrine cells. The cells have copious pale granular cytoplasm with vesicular nuclei showing prominent nucleoli (Papanicolaou $\times 480$).

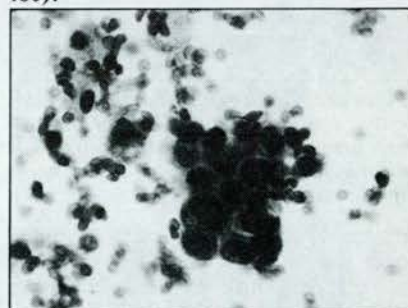


Figure 10. Cytological preparation of a cyst aspirate showing simple flattened epithelium. The cells have a small amount of cytoplasm, deeply staining nuclei and no special features. (Papanicolaou $\times 480$).



Figure 11. Cytological preparation of a cyst aspirate stained to show the specific glycolipid granules found in apocrine cells. The vesicular nuclei with prominent nucleoli, also a feature of apocrine epithelium are easily seen. (PAS diastase $\times 480$).

The relationship of cyst type to the natural history of cystic disease was then examined.

Of the 100 patients, 43 developed single or multiple cysts of flattened type, 44 patients developed single or multiple cysts of apocrine type and 13 patients had mixtures of the two types of cysts. Patients with a single cyst were more likely to have a flattened cyst and as the number of cysts aspirated in each patient increased, so the proportion of apocrine cysts also increased. (Table 1).

There were 56 episodes where patients had multiple simultaneous cysts and in 48 (88 per cent) all cysts were of the same type, ie all apocrine or all flattened. A total of 43 patients developed sequential cysts and in 32 (74

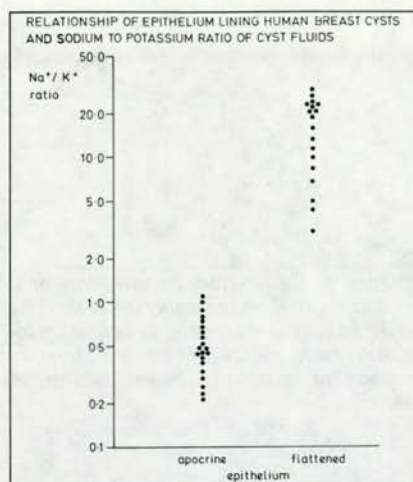


Figure 12. Ratio of Na^+/K^+ in cyst fluids where the lining epithelium of the cyst was assessed as apocrine or flattened by examination of histological or aspiration specimens.

per cent) of these, all cysts aspirated over the two year period were of the same type. Thus, patients who have multiple cysts, whether they are simultaneous or sequential usually develop cysts of one or other type.

A comparison of the frequency of further cysts in the groups of patients who presented with either apocrine or flattened cysts showed that patients who had single or multiple apocrine cysts were more than 5 times more likely to develop further cysts.

A review of 400 patients with breast cancer identified 10 patients who had a previous history of cyst aspiration where the aspirated cysts could be classified on the basis of cytology or electrolyte composition. Nine of these patients had single or multiple apocrine cysts and one had a single flattened

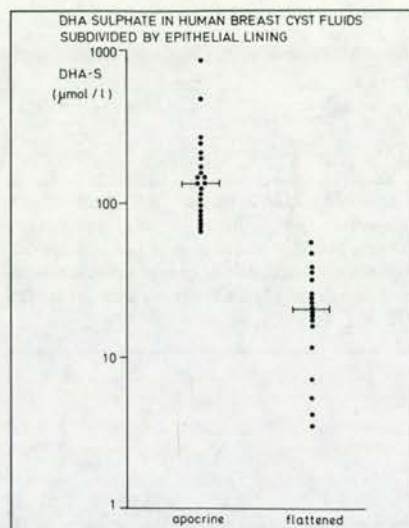


Figure 13. DHA sulphate levels in cyst fluids where the lining epithelium of the cyst was apocrine or flattened.

cyst. This ratio is markedly different to that in the general population with cystic disease (see above) and is in keeping with other observations that apocrine change occurs more frequently in populations at risk of breast cancer¹⁰⁻¹².

Conclusion

Two populations of human breast cysts can be identified. They differ in the nature of the lining epithelium and the composition of cyst fluid. Preliminary studies show that the groups are associated with clinical differences in natural history. It is also possible that these two groups also differ in their association with subsequent breast cancer. Larger numbers of patients and further follow up is necessary to confirm this.

Table 1: Comparison of the % of cysts which were classified as flattened or apocrine by electrolyte composition in group of patients who had totals of 1, 2-5 or >5 cysts aspirated over a 2 year period.

Number of cysts aspirated	Number of patients	% of cysts which were Flattened	% of cysts which were Apocrine	Ratio Apocrine Flattened
1	45	76	24	0.3
2-5	46	25	75	3.0
>5	9	6	94	15.7

Acknowledgements

J. M. Dixon is a Wellcome Surgical Research Fellow and acknowledges the support of the Wellcome Trust. Thanks are accorded to Professor A. P. M. Forrest for allowing us to investigate patients under his care.

Figures 1, 2, 3a, b and c are produced by kind permission of *Clinical Oncology*.

Figures 6 and 8-11 are produced by kind permission of the *British Journal of Surgery*.

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Literature information service in issue two

In the next issue of 'Breast News' we will be providing a literature information service with abstracts of recent academic papers on benign breast disease and related topics.

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Diary

- September 1984
Torino, Italy
International Congress on the Endocrinology of the Breast. Topics include: endocrine control of the breast, biochemistry and physiology of the breast, benign breast disease and cancer risk.
Contact: Albert Angeli, Luigi Dogliotti, Patologica Speciale Medica D, Università degli Studi di Torino, Via Genova 3, 10126, Torino, Italy.

Natural history of cystic disease: the importance of cyst type

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All breast cysts aspirated from a series of 100 patients followed for a minimum period of 2 years were classified on the basis of electrolyte composition as apocrine or flattened, this being the nature of the epithelium lining the two populations of breast cysts. Patients with a single cyst were more than 3 times as likely to have a flattened rather than an apocrine cyst. Multiple cysts, whether simultaneous or sequential in any individual patient, were usually all of the same type, and were more commonly apocrine than flattened. A comparison of the frequency of subsequent cysts in patients whose initial cysts were of either apocrine or flattened type showed further cysts were over 5 times more common in patients who presented with apocrine cysts. These observations suggest that the natural history of cystic disease is closely related to cyst type.

Keywords: Human breast cystic disease, recurrent cystic disease, apocrine epithelium

Some 7 per cent of women in the Western world develop a palpable breast cyst¹. Approximately half of these women will have a single cyst, a third develop between two and five cysts and the remainder have more than five¹. No factor has been defined which allows one to determine whether a patient will develop single or multiple cysts. Laboratory studies have shown that human breast cysts can be separated into two populations on the basis of the relative concentrations of sodium (Na^+) and potassium (K^+) in cyst fluid²⁻⁴. This variation has been shown to be due to differences in the epithelium lining the two groups of cysts⁴. The aim of the present study was to determine whether cyst type is related to the natural history of cystic disease.

Patients and methods

A prospective analysis has been carried out on 100 consecutive patients presenting for the first time with a breast cyst and followed for a minimum period of 2 years. Concentrations of Na^+ and K^+ were estimated in all cyst fluids aspirated from these 100 patients over the period of follow-up, by flame photometry (EEL model 150 flame photometer) after dilution to 1 in 200 or 1 in 400 with distilled water. Cyst fluids were classified as apocrine if $\text{Na}^+/\text{K}^+ < 3$ or flattened if $\text{Na}^+/\text{K}^+ \geq 3$ ⁴.

Statistical comparison of groups was by the χ^2 test.

Results

Over the period of study, 45 patients had only a single cyst aspirated, 46 developed between 2 and 5 cysts and 9 women had in excess of 5. A total of 247 cysts were aspirated from the 100 patients: 177 cysts were classified as apocrine and 70 as flattened on the basis of electrolyte composition. Forty-three patients developed single or multiple cysts of flattened type, 44 patients developed single or multiple cysts of apocrine type and 13 patients had mixtures of the two types of cysts.

Natural history: single or multiple cysts

Table 1 shows the percentage of cysts which were apocrine and flattened in patients subdivided according to the total number of cysts they had aspirated during the study period. Patients with a single cyst were more likely to have a flattened cyst and, as the number of cysts aspirated per patient increased, so also did the proportion which were apocrine. This is more clearly illustrated in Figure 1 where the ratio of apocrine:flattened cysts is plotted according to the total number of cysts aspirated from each patient.

Multiple cysts

There were 56 episodes where patients had multiple simultaneous cysts and in 48 (88 per cent), all cysts aspirated from individual patients were of the same type, i.e. all were apocrine or all were flattened. Forty-three patients developed sequential cysts, and in 32 (74 per cent) of these, all cysts aspirated from any one patient over the 2 year period were of the same type.

Natural history: cysts on one or more than one occasion

Of the total group of patients, 43 had cysts aspirated on more than one occasion. Overall 14 per cent of those presenting initially with flattened cysts, 72 per cent of those presenting with apocrine cysts and 50 per cent of those with mixtures of cysts, went on to develop further cysts (Table 2). Thus patients presenting with apocrine cysts developed a further cyst over 5 times more frequently than those who presented with flattened cysts. In order to determine that this was not just the case for patients with multiple cysts, patients were split into two groups, those with a single and those with multiple cysts at presentation (Table 3). The same trend existed for a greater frequency of further cysts in the apocrine group, regardless of whether patients originally had single or multiple cysts aspirated.

Discussion

Two populations of human breast cysts can be defined on the basis of the Na^+/K^+ ratio in cyst fluid²⁻⁴. By typing cysts on the basis of the electrolyte composition this study has shown that patients who develop more than one breast cyst tend to develop cysts of the same type. In any individual patient therefore multiple cysts whether simultaneous or sequential are likely to contain fluids which all have a similar composition. This

Table 1 Comparison of the percentage of cysts which were classified as flattened or apocrine on the basis of electrolyte composition in the groups of patients with 1, 2-5 and >5 cysts

No. of aspirated	No. of patients	Percentage of cysts which were classified as	
		Apocrine	Flattened
1	45	24	76
2-5	46	75	25
>5	9	94	6

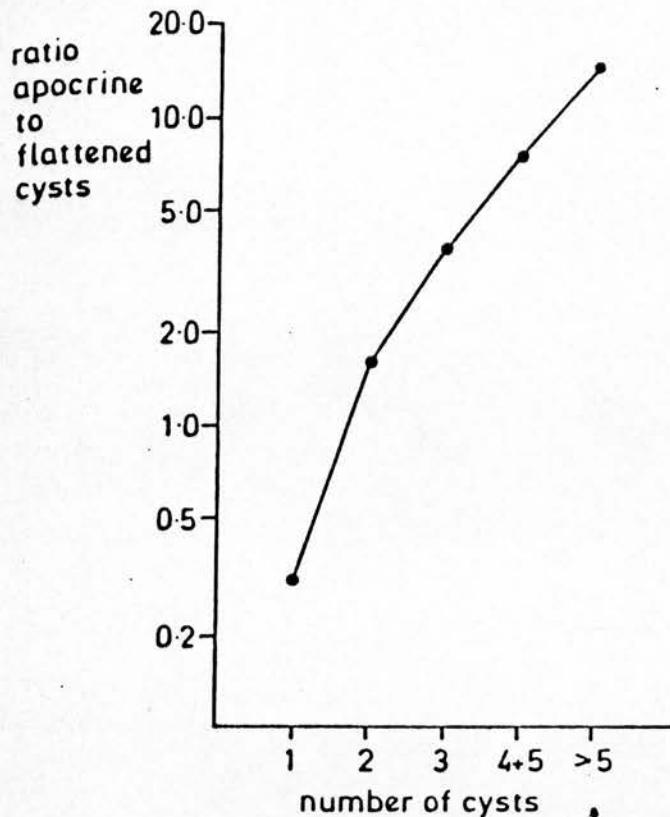


Figure 1 The ratio of apocrine:flattened cysts as classified by electrolyte composition in the groups of patients with 1, 2, 3, 4 and 5 and >5 cysts

suggests there may be a factor which determines which type of cysts an individual patient develops.

From previous studies it is known that the composition of cyst fluid relates to the nature of the epithelium lining⁴. Cysts with a low Na^+/K^+ fluid ratio (<3) are lined by apocrine epithelium and those with a high ratio (>3) are lined by flattened epithelium⁴. Apocrine epithelium is known to be an active secretory epithelium and apocrine cyst fluid contains high concentrations of secretory products²⁻⁶. In contrast flattened epithelium appears less active and fluids from cysts lined by this epithelium, more closely resemble plasma²⁻⁵. Data from this study show that patients who present with apocrine cysts are more than 5 times as likely to develop further cysts as those with cysts lined by flattened epithelium. Furthermore patients who develop more than five cysts appear to have almost exclusively apocrine cystic disease. This study then indicates a clear relationship between the natural history of cystic disease in individual patients and cyst type.

A number of treatments have been tried to reduce the numbers of cysts patients develop. From this study, it is apparent that any agent which reduces apocrine secretory activity may reduce the frequency of cyst formation. The combined oral contraceptive pill has been reported to reduce both apocrine secretion⁷ and the frequency of breast cysts⁸. Other drugs capable of reducing apocrine secretory activity may therefore also prove useful in the treatment of breast cystic disease.

It has been shown that, of patients with cystic disease, treated by aspiration, it is those who have multiple cysts aspirated who may be at increased risk of breast cancer¹. Multiple cysts have been shown in this study to be predominantly apocrine. This would suggest therefore that patients who have apocrine cysts may be more likely to develop breast cancer than those with flattened cysts. The studies which have shown that apocrine change is found more commonly in populations at risk of breast cancer⁹⁻¹³ would support such a view.

This study has shown that the majority of those patients with

Table 2 Comparison of the frequency of further cysts with groups of patients who initially presented with either flattened, apocrine or mixtures of the two types of cysts

Cyst type at presentation	No. of patients	No. developing further cysts	Percentage developing further cysts
All flattened	47	6	14
All apocrine	47	34	72*
Mixtures of 2 types	6	3	50

* Significantly greater frequency of further cysts in the apocrine than in the flattened group $P < 0.0005$

Table 3 Frequency of further cysts in patients subdivided into 2 groups: (i) according to whether they had a single or multiple cysts at presentation and (ii) according to cyst type

Cyst type at presentation	No. of patients	No. developing further cysts	Percentage developing further cysts
Single cysts			
Flattened	39	5	13%
Apocrine	30	19	63%*
Multiple cysts			
All flattened	8	1	12.5%
All apocrine	17	15	88*
Mixture of 2 types	6	3	50%

* Significantly greater frequency of further cysts in apocrine compared with flattened group $P < 0.0005$

apocrine cysts will develop further breast cysts. Estimation of either electrolyte composition or pH of breast cyst fluid allows differentiation of cysts lined by apocrine epithelium^{2,4,14}. It may also be that patients with these cysts are at greater risk of breast cancer. Electrolyte analysis or assessment of the pH of aspirated breast cyst fluid may therefore provide useful information regarding the likely natural history of cystic disease in individual patients.

Acknowledgements

Dr J. M. Dixon is a Wellcome Surgical Research Fellow and acknowledges the support of the Wellcome Trust. Thanks are accorded to Professor A. P. M. Forrest for allowing us to investigate patients under his care.

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The Relationship of Cyst Type to Risk Factors for Breast Cancer and the Subsequent Development of Breast Cancer in Patients with Breast Cystic Disease

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Abstract—The frequency of epithelial hyperplasia and papillary apocrine change in patients with palpable breast cysts lined by either apocrine or flattened epithelium has been compared. Hyperplasia of any degree, severe hyperplasia with or without atypia and papillary apocrine change were all seen significantly more frequently in patients with clinically palpable apocrine cysts. Twelve patients were identified with breast cancer with a history of cyst aspiration, in whom all cysts aspirated could be classified as apocrine or flattened on the basis of cytology or electrolyte composition of cyst fluid. Eleven patients had single or multiple apocrine cysts and one had a single flattened cyst. This represents a significantly increased preponderance of apocrine cysts as compared with that normally seen in patients with cystic disease. The mastectomy specimens of those 11 patients with a history of apocrine cyst aspiration more frequently contained hyperplastic changes and non-invasive carcinoma than age- and menopausal-matched controls who did not have a history of cystic disease. This study suggests that patients who develop cysts lined by apocrine epithelium may be at a greater risk of subsequent breast cancer than those with flattened epithelial cysts.

INTRODUCTION

SOME 7% of all women in the Western World develop a palpable breast cyst [1], and it has been reported that these women are at increased risk of breast cancer [1-3]. Pathological studies performed in patients with cystic disease have shown that the subsequent incidence of breast cancer is greatest in those women with epithelial hyperplasia associated with cysts [2-8]. The majority of women with cystic disease are not biopsied but treated by simple aspiration, and it remains uncertain which of these women are at greatest risk.

From studies of the composition of aspirated cyst fluids it has been possible to identify two populations of breast cysts on the basis of electrolyte content [9, 10]. The composition

appears to be directly related to whether the cyst is lined by apocrine or flattened epithelium [11]. The aim of the present study was first to compare the frequency and degree of hyperplasia in groups of patients with the two types of cysts and second to classify those cysts aspirated from patients who subsequently developed breast cancer.

MATERIALS AND METHODS

During the 1960s it was the practice in Edinburgh to biopsy all palpable breast lumps, even if they were considered cystic. From the years 1966 and 1967 all patients who were clinically thought to have a palpable breast cyst were identified and the histology from each patient was reviewed. The nature of the lining epithelium of two groups of cysts was noted: (i) cysts bigger than 3 mm, which have been defined previously as macrocysts [1]; and (ii) cysts bigger than 1 cm, which would make them clinically palpable. Any pathological change in each of these groups of

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biopsy specimen was recorded. Particular attention was paid to the presence of foci of apocrine change, papillary apocrine change and any hyperplasia. Hyperplasia, when present was assessed on a five-point scale using similar criteria to those of Wellings *et al.* [12]: grade I, normal; II, minimal or moderate hyperplasia; III, severe hyperplasia; IV, hyperplasia with atypia; and V, carcinoma *in situ*.

From a review of patients with breast cancer, 12 were identified who had a history of cyst aspiration and where all cysts aspirated could be classified either on the basis of cytology (five patients) or electrolyte composition (seven patients) as apocrine (apocrine cells on cytology or Na^+/K^+ ratio in cyst fluid <3) or flattened epithelium (simple epithelial cells on cytology or Na^+/K^+ ratio in cyst fluid ≥ 3). It has been previously shown that lining epithelium and electrolyte composition of cysts are directly related [11].

The mastectomy specimens of these 12 patients were reviewed, as were 12 age- and menopausal status-matched controls who did not have a history of cyst aspiration. The presence of cysts, apocrine change, hyperplasia and carcinoma *in situ* was recorded.

The χ^2 test was used to compare the frequency of pathological changes in the biopsies of patients who had cysts lined by either apocrine or flattened epithelium, and also to compare the ratio of women who had apocrine or flattened cysts in the group with breast cancer with that from a consecutive series of 100 patients treated in this department [13]. McNemar's test was used to compare the frequency of pathological changes in the pairs of matched mastectomy specimens.

RESULTS

One hundred and thirty-six patients underwent biopsy for single or multiple breast cysts during 1966 and 1967 in the Royal Infirmary, Edinburgh. In six of the biopsies no macrocyst (>3 mm) was identified and in 14 biopsies the epithelium lining the macrocyst had become detached and was not present on the sections examined. Thus

biopsies from 116 patients contained macrocysts suitable for analysis, of which 80 contained macrocysts lined only by apocrine epithelium, 30 contained macrocysts lined only by flattened epithelium and six had cysts lined by both types of epithelium. The 80 patients with apocrine cysts had a total of 183 macrocysts and the 30 with flattened cysts a total of 38. Thus on average there were 2.3 macrocysts per patient in the group with apocrine cysts, significantly more than the 1.3 macrocysts per patient in the group with flattened cysts ($P < 0.01$). Eighty-six of these 116 biopsies contained cysts which were bigger than 1 cm and which would therefore have been clinically palpable and suitably treated by aspiration; 50 biopsies contained only apocrine cysts, 30 only flattened cysts and six mixtures of the two types. There was a mean of 2.2 cysts >1 cm in the apocrine group and 1.2 cysts >1 cm in the flattened group, the difference being significant ($P < 0.01$).

A summary of the pathological changes in the biopsy specimens from the 110 patients who had either all apocrine or all flattened macrocysts and of the 80 with palpable apocrine or flattened cysts >1 cm are present in Tables 1 and 2. Separate foci of apocrine change, papillary apocrine change and epithelial hyperplasia were all significantly more common in patients with apocrine cysts. Hyperplasia of a severe degree (grade III) or with atypia (grade IV) were only seen in patients with palpable apocrine cysts.

The 12 patients who subsequently developed breast cancer had a total of 33 cysts aspirated. Three of these had only a single cyst, five had two cysts aspirated, one had three, two developed four cysts and one had nine cysts. Eleven patients had single or multiple apocrine cysts and one patient had a single flattened cyst. No patient had mixtures of the two types of cyst. This ratio of 11 apocrine:1 flattened is significantly different from that of approximately 1:1 in a consecutive series of 100 patients treated by aspiration previously reported by us ($P < 0.01$) [13]. It is also different from the 50:30 ratio of patients with palpable cysts in the histological study ($P < 0.05$).

Table 1. Comparison of the frequency of pathological changes in the surrounding breast tissue in the groups of patients who, on biopsy, were shown to have macrocysts all lined by either apocrine or flattened epithelium

Cyst epithelium	No. of patients	No. and % of patients in each group with:			
		foci of apocrine change	papillary apocrine change	hyperplasia grade II—IV	hyperplasia grade III—IV
Apocrine	80	68* (85%)	49* (61%)	50* (63%)	9† (11%)
Flattened	30	12 (40%)	8 (27%)	8 (27%)	0 (0%)

Significantly greater frequency in apocrine group: * $P < 0.005$; † $P < 0.05$.

Table 2. Comparison of the frequency of pathological changes in the surrounding breast tissue in the groups of patients who, on biopsy, were shown to have cysts >1 cm (palpable), all lined by either apocrine or flattened epithelium

Cyst epithelium	No. of patients	No. and % of patients in each group with:			
		foci of apocrine change	papillary apocrine change	hyperplasia grade II-IV	hyperplasia grade III-IV
Apocrine	50	44* (88%)	32* (64%)	39* (78%)	9† (18%)
Flattened	30	12 (40%)	8 (27%)	8 (27%)	0 (0%)

Significantly greater frequency in apocrine group: * $P < 0.01$; † $P < 0.02$.

The frequency of pathological changes in the mastectomy specimens of the 12 patients with a past history of cyst aspiration and 12 matched controls is presented in Table 3. It can be seen that cysts greater than 1 cm in size were seen only in the mastectomy specimens of those patients with a past history of aspiration of apocrine cysts. Apocrine change, papillary apocrine change, hyperplasia and carcinoma *in situ* were also seen more frequently in this group.

DISCUSSION

Controversy exists as to whether patients with cystic disease are at increased risk of breast cancer, although it is generally accepted that individuals with severe hyperplasia, epithelial atypia and papillary apocrine change are at a significantly higher risk [1-8]. Two populations of breast cysts lined by either apocrine or flattened epithelium have been described [11] and this study has clearly shown that histological risk factors for breast cancer are significantly more common in patients with clinically palpable apocrine cysts.

A number of other observations support this finding. First, in populations with a high risk of breast cancer, apocrine change is a more common finding in the breast than in populations with a low risk [14]. This may indicate that the proportion of patients with cystic disease who have apocrine cysts may vary in different parts of the world and may relate to the breast cancer risk of that area. Second, it has been reported that

women with palpable breast cysts who have histological evidence of apocrine change within the breast are more than 11 times more likely to develop breast cancer than those women with cysts without evidence of apocrine change [15]. Third, it has previously been noted that there is an association between apocrine change and epithelial hyperplasia [12, 14-17].

Having established that histological risk factors for breast cancer were more frequent in the biopsies of patients with apocrine cysts, it is of interest that this study has shown that cysts aspirated from patients who later developed breast cancer were more likely to be apocrine. Furthermore, those patients with apocrine cysts were more likely than matched controls to have within their breasts epithelial hyperplastic changes and non invasive carcinoma. It has been suggested that women who have cysts aspirated at different times develop breast cancer more often than those who have cysts on only one occasion [1]. We have previously shown that women with apocrine cysts are more likely to develop subsequent cysts [13, 18]. This, and the finding of a greater frequency of hyperplasia in the apocrine cyst group, add support to the concept that patients with apocrine cysts are more at risk of breast cancer.

The observation that individual patients with multiple cysts tend to develop cysts lined by only apocrine or only flattened epithelium has been previously reported [13, 18] and is confirmed in

Table 3. Comparison of the frequency of pathological changes in the mastectomy specimens of 12 patients with a prior history of cyst aspiration and 12 matched controls

Breasts	No. of patients	Cysts >3 mm	Cysts >1 cm	Apocrine change	Papillary apocrine change	Hyperplasia III + IV	Carcinoma <i>in situ</i>
PH* of apocrine cysts	11	11†	9†	11†	11†	11†	8†
PH of flattened cysts	1	1	0	0	0	0	0
Matched controls	12	3	0	2	1	2	2

*PH = past history.

†Significantly greater frequency of these pathological changes in mastectomy specimens of patients with apocrine cysts; $P < 0.01$ by McNemar's test.

this study. This suggests that there may be a factor, possibly genetic [19] or hormonal, which determines whether patients develop cysts lined by apocrine or flattened epithelium. This same factor, therefore, may also influence the subsequent risk of breast cancer [14].

The findings in this study relating cyst type in patients treated by simple aspiration and breast cancer cannot be directly compared with those of any other studies. This is because all previous reports relate to histopathological studies of biopsy material. As the majority of patients with cystic disease are not biopsied but treated by simple aspiration, the results of these studies are of limited relevance to clinical management. We

have previously shown that the electrolyte composition of cyst fluid is closely related to the nature of the epithelium lining the cyst [11]. As the behaviour of the two cyst types differ in relation to both natural history [13, 18] and subsequent breast cancer risk, analysis of the composition of cyst fluid may be of value in the management of patients with cystic breast disease.

Acknowledgements — J. M. Dixon is a Wellcome Surgical Research Fellow and acknowledges the support of the Wellcome Trust. Thanks are accorded to Prof. A. P. M. Forrest for allowing us to study patients under his care and to Dr T. J. Anderson, Department of Pathology, for expert pathological advice.

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pH of human breast cyst fluids

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The pH of 106 human breast cyst fluids has been measured immediately following aspiration. Values ranged from pH 6.3 to 7.8. They correlated with the ratio of Na^+ to K^+ in cyst fluid and differed significantly in the two populations of fluids which can be defined on electrolyte composition. Storage of cyst fluid for one month at -20°C resulted in an increase in pH by a mean of 0.9 of a unit. pH provides a simple method for typing cyst fluids and this may be of clinical importance as there is evidence that there are differences in the natural history and association with breast cancer in the two populations of breast cysts.

Introduction

Two separate populations of human breast cysts can be defined on the basis of the ratios of sodium (Na^+) to potassium (K^+) in cyst fluid (Bradlow *et al.*, 1981; Dixon *et al.*, 1982; Miller *et al.*, 1983). It has been shown that this variation in fluid composition reflects different types of epithelium lining the two groups of cysts (Dixon *et al.*, 1983a).

The aims of the present study were to measure the pH of fresh cyst fluid and to determine if differences in pH exist between the two populations of cyst fluids defined on electrolyte composition and to investigate the effect of storage on pH.

Patients, materials and methods

One hundred and six breast cyst fluids were aspirated from 74 patients into air tight syringes, placed on ice and pH measured within one hour, using a Corning 178 pH/blood gas analyser. Na^+ and K^+ concentrations were measured in the same fluids by flame photometry (EEL model 150 flame photometer) after dilution 1 in 200 and 1 in 400 with distilled water. Fluids were then stored at -20°C and pH was remeasured in 10 samples after 1 week and 1 month of storage.

Statistical correlation of pH and Na^+/K^+ ratio was by the Kendall rank test. Comparison of pH in the two populations of cysts was made using the Wilcoxon rank sum test.

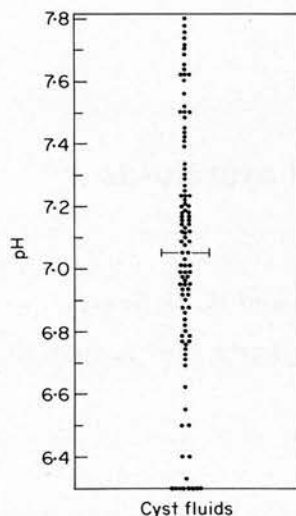


Figure 1. pH of 106 breast cyst fluids measured within 1 hour of aspiration. Horizontal bar represents mean value.

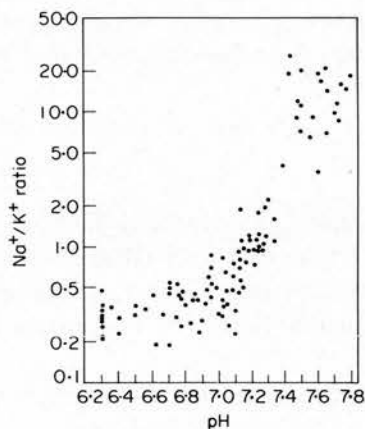


Figure 2. Correlation of pH and the Na^+/K^+ ratio of 106 breast cyst fluid.

Results

The pH values of the 106 cyst fluids ranged from 6.3 to 7.8 (Figure 1). There was significant positive correlation between pH and Na^+/K^+ ratio, $p < 0.001$ by the Kendal rank test (Figure 2). Composition of pH in fluids subdivided into different types according to Na^+/K^+ ratio showed no overlap in values between the two populations (Figure 3), the difference between the groups being significant by the Wilcoxon rank sum test ($p < 0.001$).

The effect of storage on pH in 10 cyst fluids is shown in Figure 4. After 1 week pH had increased in all fluids by a mean of 0.4 and by 1 month it had increased further by a mean of 0.9 of a pH unit over initial values.

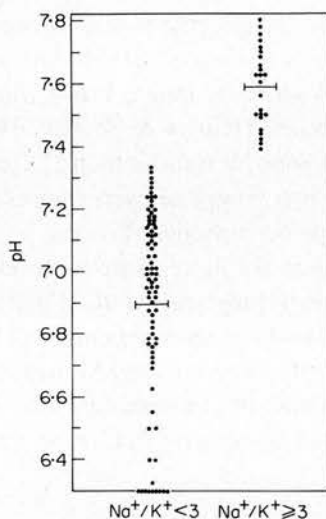


Figure 3. pH of cyst fluids separated into two populations on the basis of Na^+/K^+ ratio. The horizontal bars represents the mean values of the two groups of fluids.

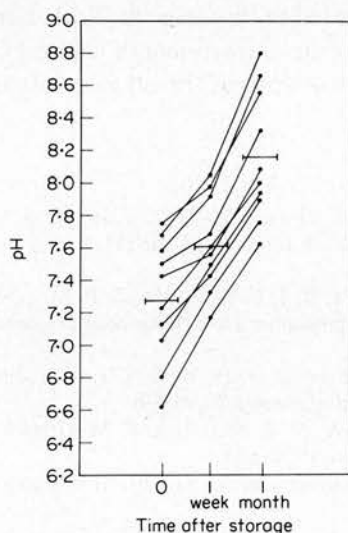


Figure 4. Effect of storage on pH in 10 cyst fluids.

Discussion

Human breast cyst fluids have been previously reported to be alkaline, with a pH range of 7.6–9.0 (Gatsy *et al.*, 1979). These measurements were however performed on stored samples and this study has shown that fresh cyst fluids are acidic or neutral and only become alkaline on storage. pH has been shown to correlate with the ratio of Na^+ to K^+ in cyst fluid and to differ in the two populations of cyst fluids defined on the basis of Na^+/K^+ ratio. Cysts with a low Na^+/K^+ ratio are thought to be lined by apocrine epithelium and cysts with a high ratio to be lined by flattened epithelium (Dixon *et al.*, 1983a). In this study apocrine cysts have been shown to have a lower

pH than flattened cysts. This is of interest as apocrine secretion occurs by expelling the contents of intracellular secretory granules (Azzopardi, 1979) and intracellular fluid has a lower pH than extracellular fluid (Waddell & Bates, 1969). Apocrine secretion from the axilla has also been noted to be acidic (Hurley & Shelley, 1960). In contrast the fluid in flattened cysts is considered to arise by transudation (Dixon *et al.*, 1983*b*). The findings of different pH values in the two groups of cysts thus correlate well with the different modes of formation which have been proposed.

It appears that patients with apocrine cysts are more likely to develop further cysts and may be at a greater risk of breast cancer (Haagensen *et al.*, 1981; Dixon & Miller unpublished). As apocrine cyst fluids are acidic, measurement of pH may be useful in the determination of cyst fluid type. Recently we have used Multistix (Ames Division Miles Laboratories Limited, England) to assess pH immediately after aspiration. This has proved a useful, simple method of identifying cyst fluid type and studies on its value are continuing.

Acknowledgements

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Protein concentrations in fluid from gross cystic disease of the breast

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Wide variations in the concentrations of IgA, IgG, lactoferrin, lysozyme and albumin were found in 96 cyst fluids obtained from 75 patients with gross cystic disease of the breast. Sedimentation coefficient determination were performed on 19 of the cyst fluids with the highest IgA concentrations in an attempt to discover the basis for the wide variation and two types of cyst fluids were found: cyst fluids that resembled external secretions such as colostrum of milk with the IgA wholly or predominantly in the 11S (secretory) form, and low concentrations of IgG and albumin, and cyst fluids that resembled serum with the IgA wholly or predominantly in the 7S (serum) form, and high concentrations of IgG and albumin. Some cyst fluids contained low concentrations of all proteins measured, and no relationship was observed between the concentrations of the proteins studied, and age, parity or menstrual status.

Introduction

Although gross cystic disease (GCD) and carcinoma of the breast share a common predisposing factor (Haagensen, 1971), the mode of formation of the fluid within the cysts in GCD is still not known. Histologically, GCD appears to be characterized by hyperplasia of acinar epithelium associated with dilatation of the terminal ducts (Schnug & Cavanagh, 1966) and it has been proposed that breast cyst fluid is a unique secretion from breast epithelial cells, possibly in response to abnormal hormone secretion (Haagensen *et al.*, 1979).

In an attempt to discover the origin of cyst fluid, we have measured the concentrations of various proteins in the cyst fluids. Concentrations of secretory IgA (11S IgA) were measured as this protein may reflect both local IgA plasma cell activity and mammary epithelial function (Lamm, 1976). Lactoferrin and lysozyme were measured

*Part of this work was performed while P. L. Yap was at the MRC Reproductive Biology Unit, Edinburgh.

as they are synthesised by glandular acini (Masson *et al.*, 1966) and epithelium (Kraus & Mestecky, 1971) respectively. In addition serum IgA (7S IgA), IgG and albumin were measured to assess the presence of serum proteins in cyst fluid.

Materials and methods

Cyst fluid

A total of 96 cyst fluids were obtained by needle aspiration from 75 women presenting with GCD. Multiple cysts were aspirated in 17 women and four women had the same cyst aspirated on more than one occasion. The age of the patients ranged from 31 to 55. Information on parity and menstrual status were available on only 69 and 71 patients respectively (Table 1). In addition, fluid was obtained from three galactocoeles in a patient following a recent pregnancy. All fluids were stored at -20°C till assay.

Quantitation of IgA

IgA was measured by double antibody RIA (Yap *et al.*, 1979), using a purified 11S IgA standard (Newcomb *et al.*, 1968). The antiserum (rabbit anti- α chain) used in all IgA measurements, unless otherwise specified, was raised in rabbits immunised with 7S IgA (Platts-Mills & Ishizaka, 1979). In addition, sucrose fractions from density gradient ultracentrifugation for sedimentation coefficient determinations were further analysed by RIA using an antiserum, prepared in rabbits immunised with 11S IgA (Dakopatts) and further adsorbed with 7S IgA. This antiserum (rabbit anti-11S IgA/FSC) only reacted with the conformational determinant of 11S IgA and the accessible determinant of free secretory component, and did not react with 7S IgA (Yap *et al.*, 1980).

Quantitation of IgG, lactoferrin, lysozyme and albumin

Single radial immunodiffusion was used to quantify IgG, lactoferrin, lysozyme and albumin using reagents and standards described previously (McClelland *et al.*, 1978). The lower limit of detection of these proteins was 100 mg/l, 50 mg/l, 90 mg/l and 50 mg/l respectively.

Sedimentation coefficient determination

Sedimentation coefficient determinations were carried out using a 25% to 45% sucrose density gradient ($236\,000 \times g$ for 3 hours) in a vertical rotor (OTD-50, Sorvall). Fractions of 0.1 ml volume were collected from the bottom of the tube, diluted and stored at -20°C till RIA with either rabbit anti- α chain, or rabbit anti-11S IgA/FSC. Two different standards were used: a serum IgA standard (Rowe *et al.*, 1972) for the measurement of IgA in ultracentrifugation fractions when the IgA detected was predominantly 7S, and the 11S IgA standard described above when the IgA detected was predominantly 11S. In a few cyst fluids, fractions from ultracentrifugal analysis were also assayed using anti-11S IgA/FSC and the 11S IgA standard. Three marker proteins were used; a non-radioactive 7S human IgG marker and radioactive ^{125}I -IgG and ^{125}I -11S IgA markers.

TABLE 1. Composition of cyst fluid and relationship to age, parity and menstrual status

	Patient (Total no.)	Cyst Fluid (Total no.)	IgA (mg/l)	IgG (mg/l)	Concentration (Mean \pm SE) Lactoferrin (mg/l)	Albumin (mg/l)
Age 31-35	5	5	155 \pm 72	495 \pm 213(1)†	747 \pm 273	1898 \pm 113
36-40	11	15	199 \pm 59	555 \pm 230(2)	1154 \pm 486	2330 \pm 1167
41-45	20	24	130 \pm 35	372 \pm 103(10)	623 \pm 111(2)	1242 \pm 496(3)
46-50	26	37	144 \pm 34	541 \pm 145(12)	1319 \pm 744(4)	1825 \pm 641(2)
51-55	12	13	238 \pm 86	827 \pm 318(4)	734 \pm 218(1)	2730 \pm 1078(1)
Not known	1	2	628 \pm 152	ND(2)	1240 \pm 0	200 \pm 0
Mean concentration			173	553(31)	1006(7)	1862(6)
Menstrual status						
Regular Periods	58	75	129 \pm 18	480 \pm 88(27)	751 \pm 119(6)	1513 \pm 365(6)
LMP 3-12 months	3	3	66 \pm 17	135 \pm 22	303 \pm 40	693 \pm 403
LMP 12-24 months	3	5	463 \pm 203	1153 \pm 669(2)	6925 \pm 6028(1)	4224 \pm 2707
LMP > 24 months	2	2	92 \pm 74	220 \pm 0(1)	1555 \pm 1245	510 \pm 440
Hysterectomy	3	4	208 \pm 146	655 \pm 474(1)	690 \pm 232	3870 \pm 2853
Not known	6	7	572 \pm 140	1006 \pm 451(2)	620 \pm 166	3360 \pm 1809
Parity						
0	25	31	151 \pm 34	522 \pm 150(9)	1638 \pm 940(5)	1989 \pm 645
1	8	10	163 \pm 67	554 \pm 233(4)	893 \pm 231	2376 \pm 1433
2	19	23	138 \pm 42	474 \pm 214(9)	959 \pm 367(2)	1308 \pm 770
3	11	13	205 \pm 69	596 \pm 159(5)	616 \pm 88	1711 \pm 829
4	4	5	97 \pm 47	545 \pm 428(2)	374 \pm 58	1516 \pm 1222
5	6	6	263 \pm 143	926 \pm 488	457 \pm 173	3642 \pm 2109
6	1	1	5 \pm 0	ND(1)	500 \pm 0	70 \pm 0
Not known	4	7	333 \pm 130	374 \pm 154(2)	686 \pm 150	1614 \pm 856

† Number of cyst fluids in which substance could not be detected (ND).

TABLE 2. Effect on cyst fluid of reaspiration of cyst after 21 days

Patient	Cyst no.	Volume (ml)	IgA concentration (mg/l)	IgG concentration (mg/l)	Albumin concentration (mg/l)
1	5	8.0	266	390	1100
	10	8.0	44	120	360
2	12	NK	42	130	550
	13	NK	32	100	300
3	18	NK	64	100	200
	19	NK	88	480	2500
4	22	5.0	275	1400	6400
	25	3.0	7	100	240

The second cyst fluid for each subject was aspirated 21 days after the initial aspiration of the cyst.

NK = not known.

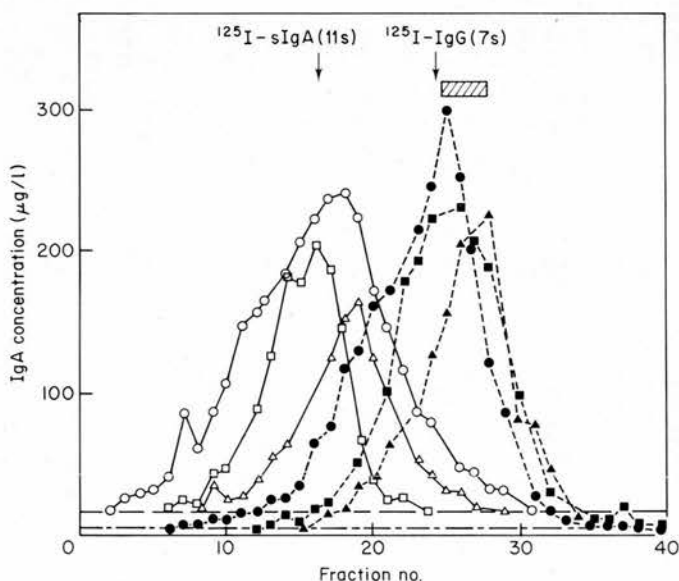


Figure 1. Sucrose density gradient ultracentrifugation to determine the sedimentation coefficient of IgA in five samples of cyst fluid 8(1), \triangle — \triangle ; 13(2), \circ — \circ ; 26, \blacksquare — \blacksquare ; 31, \blacktriangle — \blacktriangle ; 57, \bullet — \bullet (see Table 3) and a sample of fluid from a galactocoele \square — \square . A 25–45% w/v gradient was used, and 0.1 ml fractions collected from the bottom of the tube and immunoassayed using an 11S IgA standard (cyst fluid 8(1), 13(2) and galactocoele fluid) or a 7S IgA standard (cyst fluid 26, 31 and 57) and anti- α chain. The two arrows indicate the position of radioactive 11S and 7S markers, and the hatched area a non-radioactive 7S marker.

Results

IgA, IgG, lactoferrin, lysozyme and albumin concentrations in cyst fluids

Of the 96 cyst fluids investigated, all had measurable amounts of IgA, but IgG, lactoferrin and albumin could only be detected in 44 (46%), 68 (71%) and 69 (72%) fluids respectively. Lysozyme was only detected in four cyst fluids examined with concentrations ranging from 0.13 and 0.54 g/l. Concentrations of all the other proteins were

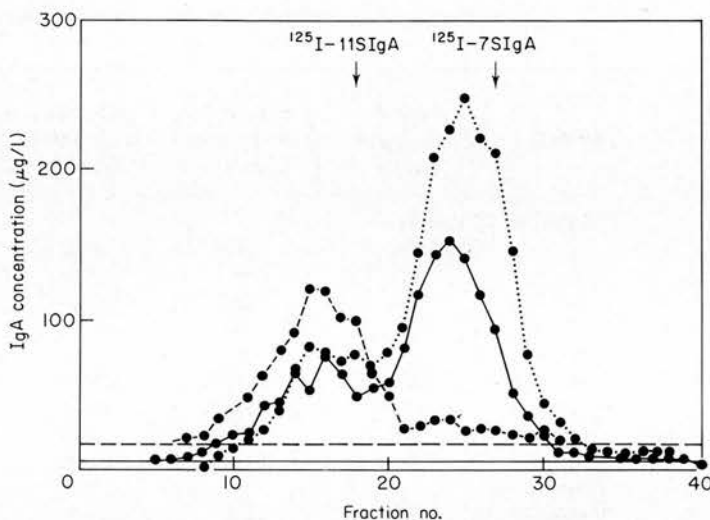


Figure 2. Sucrose density gradient ultracentrifugation to determine the sedimentation coefficient of IgA in three samples of cyst fluid 5(2), ●---●; 48, ●·····●; 26*, ●—●. Anti- α chain was used, with a 11S IgA standard (cyst fluid 5(2)) or a 7S IgA standard (cyst fluid 48, 26*). The two arrows indicate a position of radioactive 11S and 7S markers. Experimental conditions were identical to those described for Figure 1.

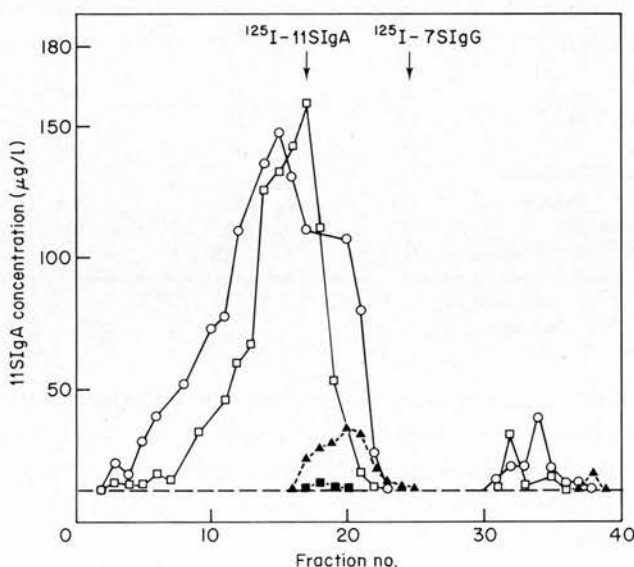


Figure 3. Sedimentation profile of three cyst fluids (13(2), ○—○; 26, ■—■; 31, ▲—▲) and fluid from galactocoele (□—□) when the fractions described in Fig. 1 were immunoassayed using anti-11S IgA/FSC (which only detects 11S IgA and free secretory component) and an 11S IgA standard.

variable and there was no obvious relationship between menopausal status, parity, age of the patients, volume of cyst fluid aspirated and the concentrations of IgA, IgG, lactoferrin and albumin (Table 1).

Multiple cysts were obtained from 17 patients, but the values again were not consistent, varying by up to 15-fold for some of the proteins measured.

TABLE 3. Classification of cyst fluids by the sedimentation coefficient of the major IgA peak

Cyst fluid no.	IgA concentration (mg/l)	IgG concentration (mg/l)	Albumin concentration (mg/l)	Lactoferrin concentration (mg/l)
IgA Peak in 7S Position				
4	796	740	6400	440
26	669	2900	6400	25000
31	639	1600	12200	640
37	664	1500	13950	400
42	867	720	1820	230
48	286	1350	8900	690
57	676	3100	17050	1010
61	939	2750	13050	300
26	334	2150	6400	1320
Arithmetic mean	652	1868	9574	3330
SE	73	300	1602	271
IgA Peak in 11S Position				
5(2)	390	ND	200	1080
8(1)	780	ND	200	1240
8(2)	475	ND	200	1240
10(1)	499	ND	70	1840
10(2)	407	100	270	1730
13(2)	776	100	120	720
18	432	ND	50	350
38(1)	308	760	1170	580
44	422	110	470	1400
23	493	ND	240	1200
Arithmetic mean	498	268	299	1138
SE	50	164	103	151
p	NS	<0.001	<0.001	NS

NS=Not significant.

ND=Not detectable.

In the three fluids obtained from the patient with galactocoeles following a recent pregnancy, mean (SE) concentrations of IgA, IgG, lactoferrin, lysozyme and albumin were 2.99 (1.73), 0.010 (0.003), 2.02 (0.57), 0.22 (0.17) and 0.60 (0.01) g/l respectively. As expected, high concentrations of IgA and lactoferrin were present in the galactocoele fluid.

Effect of reaspiration of cyst on concentrations of milk proteins

Four patients were studied on more than one occasion. In each case there was a 21-day interval between the initial and subsequent aspiration. In three of the four patients, values of IgA, IgG and albumin were lower in the second aspiration, suggesting that the aspiration procedure itself was not resulting in an increased albumin or IgG concentration (Table 2).

Sedimentation coefficient determination of IgA in cyst fluids

Sedimentation coefficient determinations were performed on the IgA present in the 19 cyst fluids with the highest IgA levels where there was sufficient material for

analysis. This was to confirm the observation of 7S IgA in a single cyst fluid (by gel filtration, data not shown) and to ensure that the correct standard was being used for the measurement of IgA by RIA in the cyst fluids.

The major peak of IgA immunoreactivity in nine of the cyst fluids studied had identical sedimentation characteristics to the 7S marker, while in the remaining ten, it was identical with the 11S marker. The IgA in fluid from the galactocoele was in the 11S position (Figure 1). Mixtures of 7S and 11S IgA immunoreactivity were seen in two cyst fluids (Figure 2).

To confirm that the immunoreactive IgA in the same position as the 11S marker was truly 11S IgA, some fractions were reanalysed, but using anti-11S IgA/FSC. The cyst fluid that had previously been found to have a 11S peak of IgA immunoreactivity again showed a 11S peak of immunoreactivity using anti-11S IgA/FSC. The fluid from the galactocoele also showed a 11S peak of IgA immunoreactivity using anti-11S IgA/FSC, confirming that in these two fluids it was the complete 11S IgA molecule that was being detected (Figure 3).

The 19 cyst fluids that were analysed by sucrose density gradient ultracentrifugation were therefore divided into cyst fluids where the IgA was wholly or predominantly either 7S or 11S. When the two categories of cyst fluids were compared, it was found that mean IgG and albumin concentrations were significantly greater in the cyst fluids where IgA was one of the 7S form than in the cyst fluids where IgA was of the 11S form. ($p < 0.001$ in all cases, Student's *t*-test; Table 3).

Discussion

Particular interest has been shown in the study of GCD because of the higher than expected incidence of breast carcinoma in patients who had previously had gross cystic disease (reviewed by Veronesi & Pizzocara, 1968). Biochemical analysis of the cyst fluids is at present incomplete: CEA (Fleisher *et al.*, 1974), various enzymes (Schwartz *et al.*, 1976), protein hormones (Srivastava *et al.*, 1977; Bradlow *et al.*, 1979) and other proteins (Haagensen *et al.*, 1979) as well as various steroid hormones (Bradlow *et al.*, 1976) including dehydroisoandrosterone sulphate (Bradlow *et al.*, 1981a), oestriol conjugates (Raju *et al.*, 1977) and a progesterone binding component (Pearlman *et al.*, 1973) have all been found in cyst fluids, but the origin of the fluid within the cyst remains unresolved.

Cyst fluids from patients with GCD were therefore analysed to investigate the possibility that the cyst fluid might either reflect the synthetic activity of the surrounding breast tissue, or the transit of serum proteins into the cyst. It was thought that this might lead to an understanding of the mechanism of cyst formation.

The results of this study show that a wide range of concentrations of IgA, IgG, lactoferrin and albumin can be detected in cyst fluids. Although the rate of detection was a reflection of the sensitivity of the assay method used, the high detection rate for lactoferrin, which is not normally detectable in serum suggests that the cyst fluid probably does indicate the synthetic activity of the surrounding breast epithelium. Interestingly, lactoferrin concentrations in cyst fluid were about one fifth the concentration in milk (McClelland *et al.*, 1978).

Density gradient ultracentrifugation was used to characterise the IgA detected. Of

19 cyst fluids analysed by ultracentrifugation, half contained IgA that was wholly or predominantly of the 11S type, and the remaining half contained IgA of the 7S type. The latter cyst fluids also contained high concentrations of two other serum proteins, IgG and albumin, suggesting that components of serum were entering the cyst by some unknown route. In these cyst fluids, the mean concentrations of IgA, IgG and albumin were approximately four-fold lower than the corresponding concentrations in adult serum. The high concentrations of 11S IgA present in some cyst fluids suggested that local mammary synthesis of 11S IgA was occurring, as little 11S IgA is present in serum (Waldman *et al.*, 1970).

Insufficient data was present to draw any firm conclusions about the relationship between protein concentrations, and the various clinical parameters recorded but the results of this study indicate that breast cysts may be more heterogenous than previously thought, and that two or more types of cysts may occur in gross cystic disease of the breast. One type of cyst contains fluid reflecting the synthetic activity of the surrounding mammary epithelium and sub-epithelial plasma cells, while another type of cyst appears to contain serum components. The suggestion that two types of cysts may exist has been reported by Barlow *et al.* (1981*b*). An alternative explanation is that the fluid contents of the cyst may be changing over a period of time, and that the differences in protein concentration we may have observed do not reflect different types of cysts, but possibly similar cysts at different stages in their formation. Further studies involving reaspiration studies of the same cyst are therefore required, as are studies of whether the determination of protein concentrations is of any clinical importance to the patient.

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Hormonal Correlates of Apocrine Secretion in the Breast^a

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INTRODUCTION

High, but variable levels of the androgen conjugate dehydroepiandrosterone (DHA) sulfate are present in breast fluids and tumors.¹⁻⁶ Evidence will be presented that these relate to apocrine activity within the breast. This conclusion is based on (1) measurements of DHA sulfate in secretions obtained by nipple aspiration and breast fluids derived from micro- and macrocysts and (2) the distribution of androgen conjugates within normal lobules, cysts, and cancerous lesions of the breast.

METHODS

Radioimmunoassay was performed on all breast fluids after dilution with phosphate buffer using the method of Buster and Abraham⁷ and employing an antibody whose specificity has been previously described.⁸ The antibody crossreacts primarily with DHA and its sulfate, but also shows significant crossreactivity with DHA glucuronide and epiandrosterone conjugates.

Gas-liquid chromatography-mass spectrometry was performed on extracts of solvolyzed breast secretions, as described previously,² and involves analysis as the tertiary butyl dimethyl silyl (TBDMS) derivatives.

Immunohistochemical studies were performed on frozen sections (3-5 μ) of fresh tissue. These were incubated successively in 10% normal rabbit serum, the antisera to DHA sulfate (raised in sheep) diluted in 10% normal rabbit serum and 5% human serum (containing undetectable levels of DHA sulfate), and peroxidase-conjugated rabbit anti-sheep antibody. Visualization was achieved with the use of diaminobenzidine, which turns brown when peroxidase is present.

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RESULTS

Normal Breast Secretions

Breast secretions were obtained by nipple aspiration, from women volunteers who had no clinical evidence of breast abnormality; aspiration was done by applying a slight negative pressure to the nipple by means of a syringe attached to a cup.⁴

The levels of DHA sulfate in 100 secretions obtained from 61 women were measured by radioimmunoassay. The results are presented in FIGURE 1 and show an enormous range of values from 20 to 2250 $\mu\text{mol/L}$ (median value, 270 $\mu\text{mol/L}$). Data obtained from gas-liquid chromatography-mass spectrometry confirmed that the material crossreacting with the antibody in the radioimmunoassay was a conjugate of DHA. The major component of solvolyzed extracts of breast secretions on gas-liquid chromatography had a mass spectrum closely resembling that of authentic TBDMS-

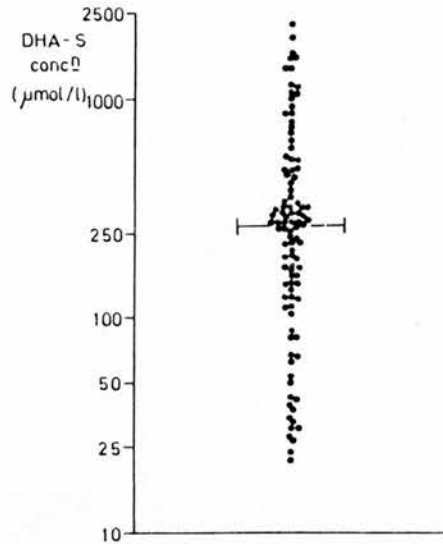


FIGURE 1. Levels of DHA sulfate in breast secretions from normal women. Horizontal line represents the median value.

DHA. Levels of DHA were also similar, whether measured by radioimmunoassay or by gas-liquid chromatography-mass spectrometry.

Peripheral blood was taken by venipuncture immediately before aspiration of breast secretions in 20 normal women. In each case, the level of DHA sulfate was higher in breast secretion than in plasma. Although the average value was more than 100-fold higher in secretions than in plasma, the breast secretion:plasma ratio for DHA sulfate varied from 7 to 380 between individual women (TABLE 1).

In an attempt to localize DHA sulfate within normal breast, immunohistochemical studies were performed on frozen sections of material from bilateral reduction mammoplasties performed in two patients. The secretion in normal breast lobules which stained pink with hematoxylin and eosin contained material crossreacting with the antibody for DHA sulfate (FIG. 2). There was little or no apparent staining within the cytoplasm of normal breast lobular epithelium. Secretions in breast ducts also

TABLE 1. Levels of DHA-Sulfate ($\mu\text{mol/L}$) in Breast Secretions and Plasma from Normal Women

Subject	Breast Secretion ^a	Plasma	Ratio
1	1,028	3.26	315
2	168	1.75	96
3	318	1.92	165
4	194	2.43	80
5	567	4.21	135
6	306	1.94	158
7	396	2.43	163
8	23	3.29	7
9	867	2.28	380
10	1,482	7.05	210
11	122	1.74	70
12	249	2.00	125
13	828	5.71	145
14	165	2.04	81
15	173	3.08	83
16	277	1.26	220
17	118	2.36	50
18	284	2.02	140
19	192	3.97	48
20	299	3.05	98

^aWhere more than one secretion was obtained per subject, the values have been averaged.

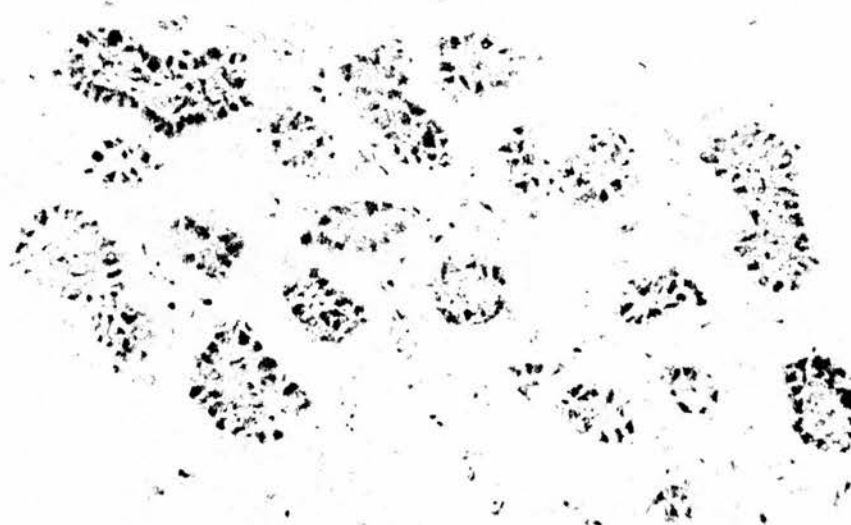


FIGURE 2. Normal human breast lobule stained by the immunoperoxidase technique to show the presence of DHA sulfate. (Frozen section; magnification $\times 40$).

stained heavily in the peroxidase studies; some staining was localized in ductal-epithelium.

Microcysts

Microcysts were arbitrarily distinguished from macrocysts by being impalpable within the breast and smaller than 3 mm in diameter. Forty microcysts were identified in eight biopsy specimens for benign breast disease and 14 mastectomy specimens for breast cancer. The fluid from microcysts was collected by aspiration into fine glass tubing. All samples contained high levels of DHA sulfate as measured by radioimmunoassay, the concentrations being similar to those in breast secretions and much in excess of those in plasma (FIG. 3).

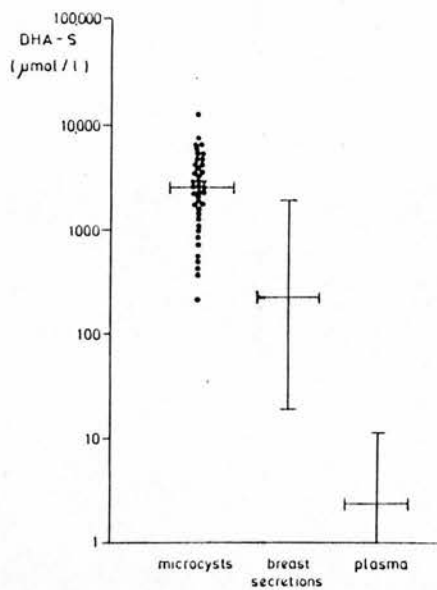


FIGURE 3. Levels of DHA sulfate in human breast microcysts. Horizontal lines represent median values; vertical lines show range of values for either breast secretions or plasma from normal women.

After collection of the fluid, the microcysts were examined histologically and subjected to immunoperoxidase staining for DHA sulfate. Examples of the results are shown in FIGURE 4. The microcysts were lined by epithelium which showed apocrine features and which also stained deeply positively with the anti-DHA sulfate antibody.

Macrocysts

Levels of DHA sulfate in fluids obtained from palpable breast cysts varied enormously from 2 to 700 $\mu\text{mol/L}$. (FIG. 5). This large concentration range means that



FIGURE 4. Human breast microcyst epithelium stained by the immunoperoxidase technique to show the presence of DHA sulfate. (Frozen section; magnification $\times 400$; reduced by 20%.)

certain cyst fluids have values of DHA sulfate similar to those in plasma while others have levels similar to those in breast secretions. Factors that might account for this large variation were examined. No significant relationships were detected between DHA sulfate and cyst fluid volume or age, parity, or menopausal status of the patients. However, there were significant correlations between DHA sulfate and other constit-

FIGURE 5. Levels of DHA sulfate in human breast macrocysts. Horizontal lines represent median values; vertical lines range of values for either breast secretions or plasma from normal women.

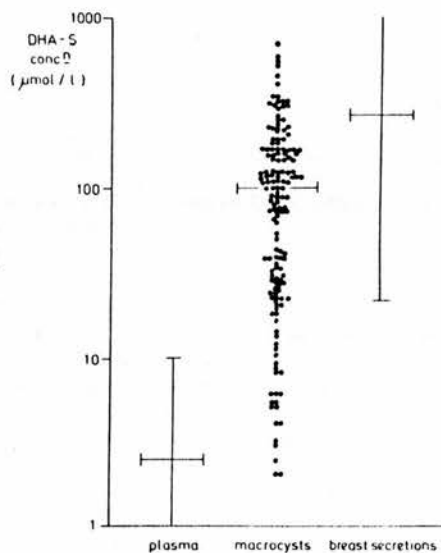


FIGURE 7. Distribution of the ratios of $[\text{Na}^+]$ to $[\text{K}^+]$ in fluids from macrocysts. Two subpopulations are apparent with either a low (<4) or high (>4) ratio.

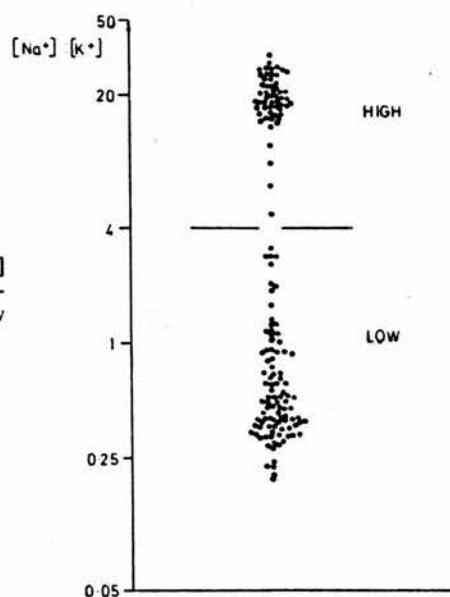
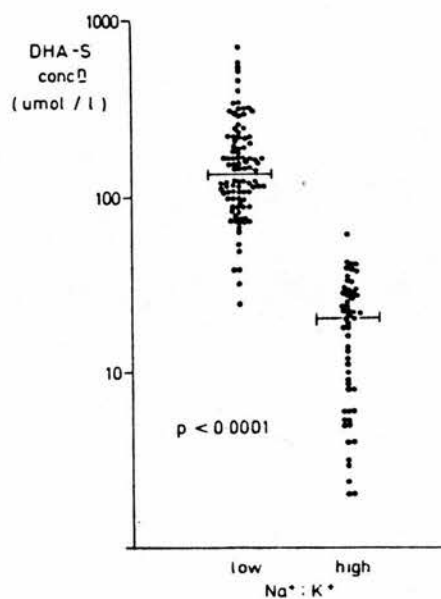


FIGURE 8. Comparison of DHA sulfate levels in human breast cyst fluids with either a low (<4) or high (>4) $\text{Na}^+:\text{K}^+$ ratio. Difference between the groups is significant by the Wilcoxon rank test ($p < 0.0001$).



Breast Carcinomas

Frozen sections from 20 cases of human breast cancer were processed for hematoxylin and eosin histologic examination and for immunohistochemical study using antibody against DHA sulfate. The hematoxylin and eosin-stained sections were graded for apocrine differentiation and the immunohistochemical sections for the degree of staining for DHA sulfate. There was a highly significant correlation between the presence of apocrine features within the breast tumors and the degree of staining for DHA sulfate (TABLE 2). Furthermore, within the tumors displaying apocrine differentiation, the staining for DHA sulfate was particularly localized in cells having apocrine features, as shown in FIGURE 12.

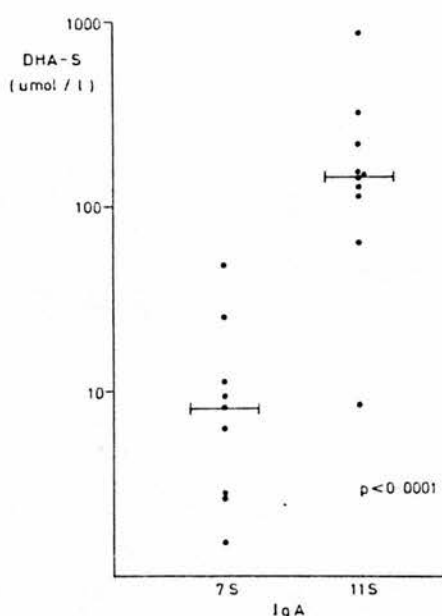


FIGURE 9. Comparison of DHA sulfate levels in human breast cyst fluids subdivided according to their predominant type of IgA. Difference between the groups is significant by Wilcoxon rank test ($p < 0.0001$).

DISCUSSION

Evidence has been presented that levels of DHA sulfate in normal, benign, and malignant breast are related to the apocrine activity of the tissue. In fluids from benign breast cysts, there are strong positive correlations between levels of DHA sulfate and K^+ (normally an intracellular cation), 11S IgA (a marker of secretory activity), and the presence of an apocrine epithelium cyst lining. Since no significant relationships were evident between DHA sulfate and other variables such as cyst fluid volume, and menopausal status, age and parity of the women, these data are compatible with the notion that in the fluids that contain high levels of DHA sulfate these high levels are produced as a result of apocrine activity of the cyst lining. In contrast, those fluids

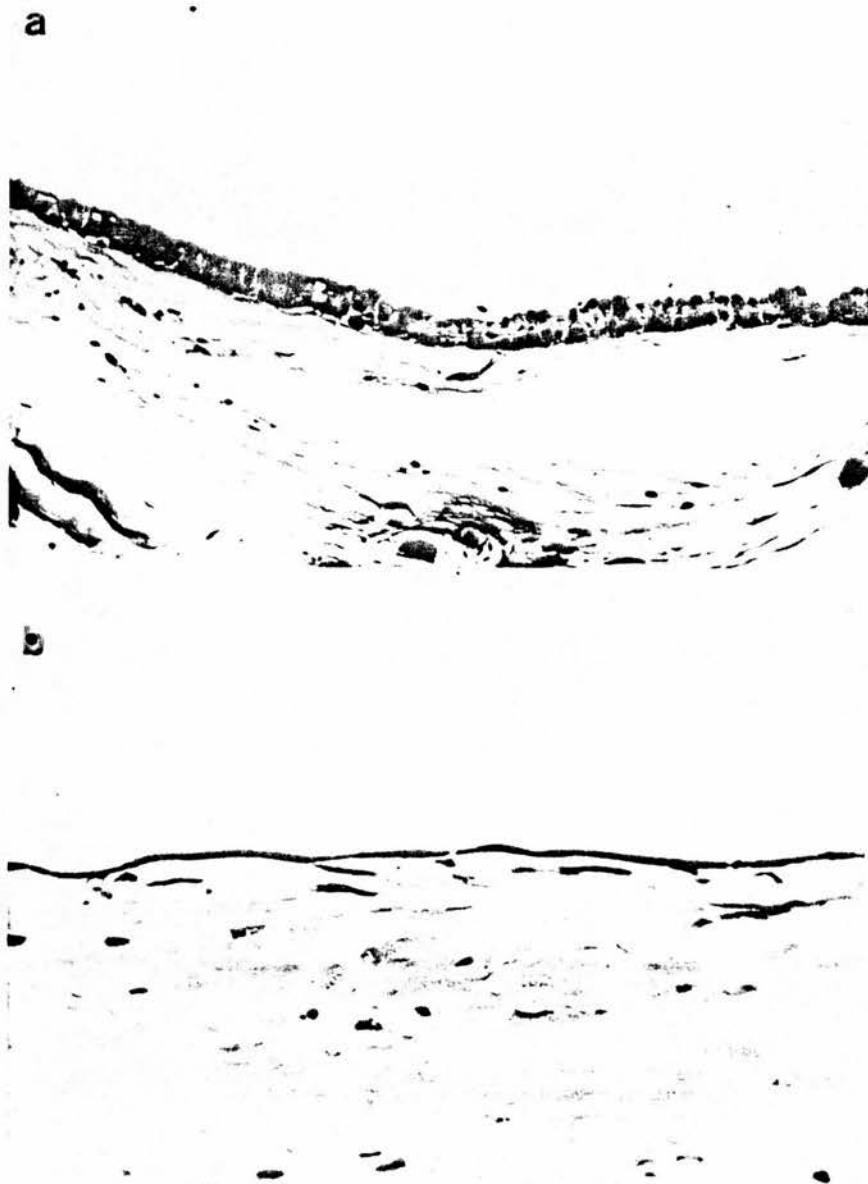


FIGURE 10. Histologic specimens of the epithelium lining human breast cysts. (a) Apocrine epithelium (hematoxylin and eosin stain; magnification $\times 120$). (b) Flattened epithelium (hematoxylin and eosin stain; magnification $\times 240$).

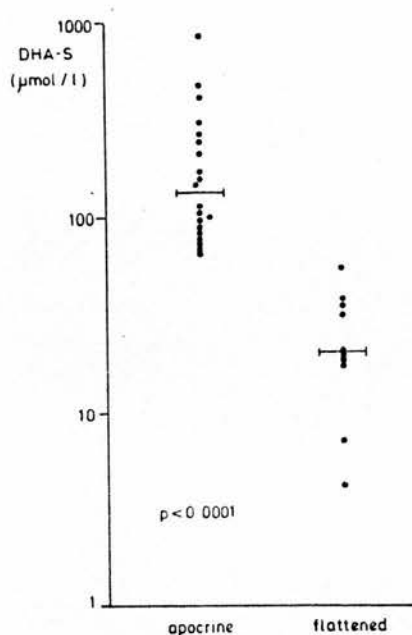


FIGURE 11. Comparison of DHA sulfate levels in fluids from cysts lined either by apocrine or flattened epithelium.

with low levels of DHA sulfate probably result from a more passive non-apocrine process. It is therefore consistent that microcysts which, in our limited series, were wholly lined by apocrine epithelium should show the highest concentrations of DHA sulfate.

This relationship between DHA sulfate and apocrine activity was not restricted to benign apocrine epithelium and, in a small group of cancerous breast tumors, there was a significant positive correlation between degree of apocrine activity and staining for DHA sulfate. These immunohistochemical studies indicated that in cases of breast cancer showing apocrine differentiation, the DHA sulfate is primarily associated with apocrine cells.

TABLE 2. Relationship Between Degree of Apocrine Differentiation and Degree of Staining with DHA Sulfate Antibody Peroxidase Technique

Degree of Apocrine Differentiation	Degree of Staining for DHA Sulfate ^a		
	0	+	++
Absent	6	0	0
Minimal	1	7	1
Moderate	0	1	4

NOTE: The correlation between apocrine differentiation and staining was statistically significant using the Kendall rank correlation test $T = 0.85$ ($p < 0.001$).

^aDegree of staining for DHA sulfate was classified as 0 if there was no apparent staining, + if only occasional cells stained, and ++ if there was a substantial proportion of cells showing the stain.

Further support for the concept that DHA sulfate may be a marker of apocrine activity is the report of high concentrations of the steroid in other apocrine secretions such as apocrine sweat from axillary glands.¹⁰

High concentrations of DHA sulfate were demonstrated in breast secretions obtained by nipple aspiration from breasts without clinical abnormality. Histochemical staining for DHA sulfate also indicated that the eosinophilic secretion within the lumen of normal lobules contains large amounts of androgen conjugate. By inference, these observations may be taken as evidence of apocrine activity within normal breast cells. It ought to be emphasized, however, that if these lobules represent the deep-

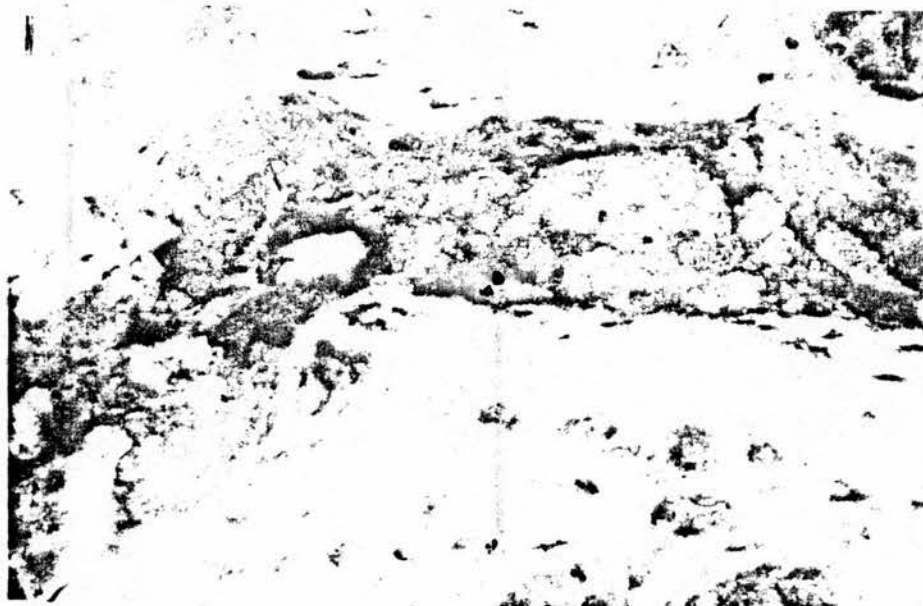


FIGURE 12. Tumor with marked apocrine differentiation stained by the immunoperoxidase technique to show the presence of DHA sulfate. (Frozen section; magnification $\times 100$.)

seated source of the secretions obtained by nipple aspiration, modification of the composition of the fluids is likely to occur by selective reabsorption or further secretion during the passage through the ductal system. The constituents of nipple aspirates would therefore not necessarily reflect the activity of normal breast lobules.

The origin, biological significance, and clinical relevance of DHA sulfate within apocrine cells of the breast still remain to be elucidated. It is possible that DHA sulfate could be synthesized locally from other steroid precursors, cholesterol or cholesterol sulfate, but such biosynthetic capacity has never been convincingly demonstrated within the breast. It seems more likely that DHA sulfate is selectively concentrated from plasma where it circulates in large amounts for a blood-borne hormone and is almost exclusively derived from the adrenal cortex. Bradlow *et al.*¹¹ found that among a series of radioactively labeled hormones administered to women with gross cystic disease of the breast, DHA sulfate alone accumulated in breast cyst fluids; this finding is consistent with a mechanism of selective concentration.

DHA sulfate and other closely related androgen conjugates have little biological activity per se^{12,13} but they may be pre-hormones of steroids with more potent properties.^{14,15} There is evidence that breast tissues may utilize DHA sulfate as a precursor,¹⁶ and pathways have been traced that lead to both active androgens and estrogens.¹⁶⁻¹⁸ Cancerous breast tumors with marked apocrine differentiation display a high potential to produce 5 α -reduced androgens.¹⁹ These steroids have potent androgenic activity and it is interesting that apocrine activity itself is stimulated by androgenic steroids.²⁰ It may also be relevant that, in addition to androgen conjugates, estrogen conjugates are present in high concentrations in breast cyst fluids, but these are not concentrated from the circulation.²¹ The suggestion is that estrogen conjugates may be derived locally within the cyst by aromatization from androgen precursors such as DHA sulfate. Unpublished data from this department indicate a strong correlation between concentrations of DHA sulfate and estrone sulfate in breast cyst fluids.

Several recent studies²²⁻²⁴ have suggested that the presence of apocrine activity within the breast is a predisposing factor for breast cancer. If this is so, then the measurement of DHA sulfate within breast fluids and tissues may form a useful marker to determine those at increased risk.

ACKNOWLEDGEMENTS

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Platelet-associated proteins in human breast cyst fluids

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Summary

Thrombospondin, β -thromboglobulin and platelet factor 4 were measured by radioimmunoassay in fluids from 50 human breast cysts. β -Thromboglobulin concentrations were < 4–330 ng/ml (median 13.5 ng/ml), while normal plasma contained 14–80 ng/ml. Cyst fluids contained 8–225 ng/ml platelet factor 4 with a median of 25 ng/ml (range in plasma 6.5–29 ng/ml). Thrombospondin levels in cyst fluids were 65–55 000 ng/ml (median 2 500 ng/ml) and 92% contained concentrations above the plasma range (58–215 ng/ml). When cyst fluids were classified by their electrolyte composition, group II cysts had similar levels of platelet factor 4 but significantly higher levels of β -thromboglobulin and thrombospondin than group I. From the remarkably high concentrations of thrombospondin within cyst fluids and the differences between cyst types, it seems that either the protein is synthesised locally or selectively accumulated. Increased platelet activation is unlikely to be the source of the high thrombospondin levels in group II cysts.

Introduction

Cysts are the most common benign lesion of the breast [1] and women with cystic disease appear to have an increased risk of developing breast cancer [1,2]. Despite this, comparatively little is known about the composition of cyst fluid. Recently, however, it has been shown that cyst fluids may contain unusually high concentrations of steroid conjugates [3,4] and specific proteins [5,6]. It is also possible to

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classify cyst fluids into two major sub-groups according to electrolyte composition [7] and the histological nature of the lining epithelium [8].

Our unpublished observations that milk contains high concentrations of thrombospondin prompted the present investigation into platelet associated proteins in cyst fluids. Thrombospondin is a glycoprotein which was first isolated from the extracellular medium after thrombin treatment of platelets [9], but has since been shown to be synthesised and secreted by endothelial cells [10,11], smooth muscle cells [12] and fibroblasts [13]. On the other hand, β -thromboglobulin (β -TG) is a platelet-specific protein [14], and platelet factor (PF4) has been identified only in platelets [15] and mast cells [16]. In this paper, we report the presence of thrombospondin, β -TG, and PF4 in breast cyst fluids, and their distribution in the major subpopulations of cysts.

Materials and methods

Fifty breast cyst fluids were obtained by needle aspiration from 45 patients. These represented aspiration of a single cyst in 42 women and multiple cysts in 3 subjects. The fluids were centrifuged at $600 \times g$ for 10 min and the supernatant stored at -20°C until assayed.

Concentrations of Na^+ and K^+ were measured by flame photometry (EEL model 150 flame photometer) after dilution 1 in 200 and 1 in 400 with distilled water. Fluids were then classified as group I if the concentration ratio of Na^+ to K^+ was less than 4 (25 fluids) or group II if the ratio was > 4 (25 fluids).

Thrombospondin was measured by radioimmunoassay essentially as described by Dawes et al [17]. Polyclonal antibody was raised in New Zealand white rabbits using thrombospondin purified from human platelets as immunogen. Thrombospondin was radiolabelled with ^{125}I using the chloramine T technique of Greenwood et al [18]. Assays were carried out in 0.05 mol/l phosphate buffer pH 7.4 containing 2% horse serum and 1% Tween 20. Each tube contained antiserum (50 μl of a concentration sufficient to bind 50% of the tracer), [^{125}I]thrombospondin (50 μl of 10 ng/ml) and standard or sample, in a final volume of 250 μl . After incubation overnight at 20°C , free thrombospondin was separated from that bound to antibody using donkey anti-rabbit IgG antiserum (Scottish Antibody Production Unit) coupled to Sepharose 4B.

β TG and PF4 were purified from human platelets by affinity chromatography on heparin-Sepharose. Radioimmunoassays for these proteins are similar to that for thrombospondin and are described by Bolton et al [19] and Dawes et al [20]. Antisera for all three radioimmunoassays were as described in previous publications. In all cases cyst fluids gave parallel dilution curves to standards and serum samples, indicating that the proteins in the different fluids were antigenically indistinguishable using these polyclonal antisera.

Results

The levels of β TG, PF4 and thrombospondin in cyst fluids are shown in Figs. 1 and 2. All cyst fluids contained measurable amounts of each protein apart from one

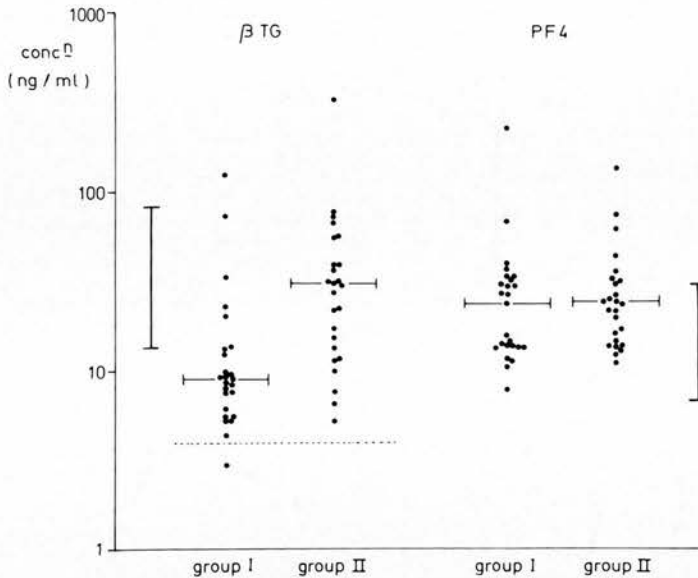


Fig. 1. Levels of β TG and PF4 in cyst fluids subdivided according to electrolyte composition (group I $\text{Na}^+ : \text{K}^+ < 4$, group II $\text{Na}^+ : \text{K}^+ > 4$). Solid lines represent median values for the sub-groups, broken line the level of sensitivity and vertical lines the range for each protein in blood plasma. There was a significant difference in β TG between the two sub-groups by Wilcoxon Rank test ($p < 0.001$) but no significant difference in PF4 levels.

which had undetectable levels of β TG. There was a wide range of values for β TG (< 4 to 330 ng/ml). Twenty-four of the 50 fluids (48%) contained concentrations within the reference range for normal blood plasma of 14–80 ng/ml (11); all but 2 of the remainder possessed lower levels. Values for PF4 in cyst fluids ranged between 8 and 225 ng/ml (median value 25 ng/ml). In 34 fluids, concentrations fell within the reference range of 6.5–25 ng/ml for normal plasma (11), the remaining 16 fluids possessed levels in excess of those in plasma. Whilst there was a very large range of values for thrombospondin in cyst fluids, the concentrations were usually greatly in excess of those in normal plasma (Fig. 2). The median value of 2500 ng/ml for thrombospondin for all cyst fluids was about 25-fold higher than that in plasma (range 57–216 ng/ml (9)).

The effects of sub-dividing the cyst fluids according to their electrolyte class is shown in Fig. 1 for β TG and PF4 and in Fig. 2 for thrombospondin. Concentrations of β TG were significantly higher ($p < 0.001$) in group II cysts compared with group I. However, whilst the median value for β TG in group II cysts was over 3-fold higher than in group I, there was a large overlap in values between the two groups. No significant difference was apparent in PF4 between the sub-classes of cysts and the median value was similar in both groups. In contrast, there was a highly significant difference in concentrations of thrombospondin between the two groups

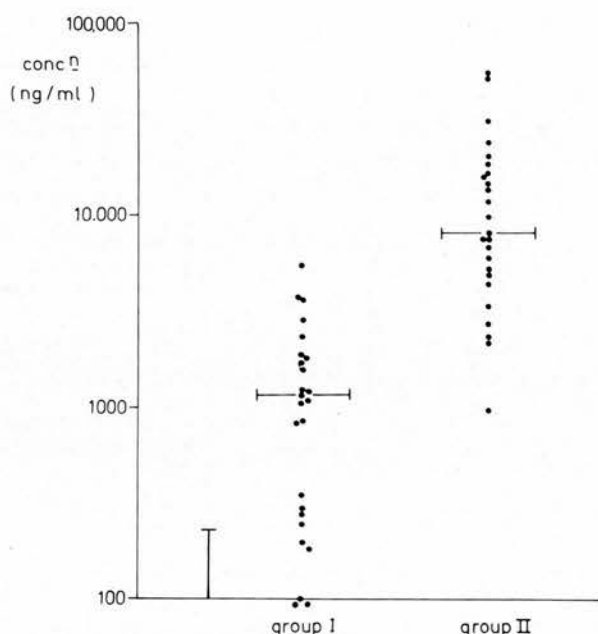


Fig. 2. Levels of thrombospondin in cyst fluids subdivided according to electrolyte composition. Horizontal lines represent median values for the sub-groups and the vertical line the range for blood plasma. There was a significant difference between the groups by Wilcoxon Rank test ($p < 10^{-6}$).

of cysts. The median value in group II cysts was 7-fold higher than in group I and all fluids containing in excess of 6 000 ng/ml were from group II.

Discussion

These results are the first report of the presence of several platelet-associated proteins in breast cyst fluid. Whilst levels of β TG were in general similar to or lower than those in normal plasma, concentrations of thrombospondin were markedly higher in most cyst fluids, and raised levels of PF4 were also detected in a proportion of fluids.

The presence of PF4 concentrations above those found in normal plasma is interesting. It is unlikely to be derived directly from platelets, as equivalently elevated levels of β TG, which is released from the platelet at the same time as PF4, were not observed. Diffusion from the plasma is equally unlikely, for PF4 circulates as a large complex which is present in plasma at lower concentrations than β TG in both normal and clinical situations [20]. However, it is thought that PF4, unlike β TG, may be cleared from the circulation by binding to heparan sulphate on the vascular endothelium [21], and it could be transferred to cyst fluids by this route. Alternatively it may derived from tissue mast cells, which do contain PF4 but not β TG [16]. This raises the question of whether mast cells are activated in cystic disease.

The thrombospondin levels in cyst fluids also cannot be explained on the basis of platelet release. Indeed, in some cases they are higher than the concentrations (11.5–30.75 $\mu\text{g/ml}$) achieved in serum. We have not excluded the possibility that the material in cyst fluid is not identical to platelet thrombospondin but, this seems unlikely. The two are antigenically indistinguishable using our polyclonal antisera, and are currently being tested with a range of monoclonal antibodies raised against platelet thrombospondin.

It is of great interest that thrombospondin is not only present at higher concentrations in cyst fluid than plasma but additionally shows highly significant differences in levels between the two major sub-groups of cyst fluid. Thus group II fluids, which are characterised on the basis of containing high concentrations of Na^+ but low levels of K^+ , possessed higher concentrations of thrombospondin and βTG than group I fluids which have comparatively higher K^+ and lower Na^+ . Group II fluids also have the 7S non-secretory form of immunoglobulin A [22] and are invariably lined by thin attenuated epithelial cells [8]. Because of this, it has been inferred that the contents of such fluids accumulate in a passive manner [22,23], possibly as a transudate from blood, as suggested above for βTG . However, the extraordinarily high levels of thrombospondin in group II cyst fluids would suggest that either there is an active but selective accumulation process against a concentration gradient or that the protein is synthesised locally within the breast, possibly by the cells of the cyst wall. The active cell type has yet to be identified, but is unlikely to be the apocrine cells as there are lower levels of thrombospondin in group I cysts which are usually lined by active apocrine cells [8] and whose fluids also possess the 11S secretory form of immunoglobulin A [22]. Concentrations of thrombospondin in saliva are within the normal plasma range (unpublished results), supporting the conclusion that thrombospondin is not a simple marker of apocrine activity. The accumulation of platelet-associated proteins in the breast is not restricted to cystic fluids and our preliminary results indicate that other breast fluids such as galacto-heic secretions and those obtained by nipple aspiration from non-lactating women also contain very high concentrations of thrombospondin.

The significance of high concentrations of this particular protein within the breast is unknown. It is thought to form part of the matrix involved in platelet aggregation, and may therefore be associated with thrombus formation. However, as it becomes increasingly clear that thrombospondin is widely distributed among cell types and body fluids, more general functions for this protein must be sought. The concentrations we have found in breast fluids are much higher than elsewhere; the breast may therefore be the ideal tissue in which to investigate these functions.

Acknowledgements

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An analysis of the content and morphology of human breast microcysts

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The composition of 40 human breast microcysts, dissected from eight biopsy and 14 mastectomy specimens has been analysed. All contained high concentrations of the androgen conjugate, dehydroepiandrosterone sulphate, the median value being more than 1000 times that of plasma. In ten cysts sufficient fluid was available for cationic analysis and in all the potassium concentration exceeded that of sodium. This composition suggests an apocrine derivation of the constituents of breast microcysts. This is substantiated by the finding of PAS diastase positive granules in the epithelium lining all 40 microcysts. These findings indicate that there is a single population of breast microcysts which are lined by epithelium with apocrine secretory activity.

Key words: Fibrocystic disease of breast; hormones; cations; apocrine epithelium.

Introduction

Human breast microcysts which, by definition, measure less than 3 mm in maximum dimension are considered to be the precursors of clinically palpable breast macrocysts.^{1,2} Two populations of human breast macrocysts have now been identified—these have a different content and are lined by different types of epithelium.³⁻⁶ Little, however, is known of the content of human breast microcysts and, in particular, it is not known if there exists a single or a dual population. It was the aim of this study to analyse the content of human breast microcysts and to determine the nature of their epithelial lining.

Materials and methods

Forty microcysts were dissected from eight biopsy and 14 mastectomy specimens before fixation using a dissecting microscope. In all eight biopsies the major pathology was cystic disease. A total of 16 macrocysts were present in these eight specimens, three contained only flattened cysts, three only apocrine cysts and two had mixtures of both types.

The 14 mastectomies were for malignancy. Each also contained macrocysts, ten only apocrine, one only flattened and three mixtures of two types. The 40 microcysts were a random sample of the total of 92 microcysts in these specimens. The fluid within the microcysts was collected after puncture in calibrated capillary tubes to measure the volume (range 0.5–20 µl) before dilution in distilled water. They were stored at –40°C prior to analysis. The cyst wall was fixed in 10% formal saline and submitted for histological processing, staining and serial sectioning.

Analysis of microcyst fluid

The DHA sulphate concentration in each of the 40 microcyst fluids was estimated by radioimmunoassay using an antibody previously described.⁷

Estimations of the concentrations of sodium (Na⁺) and potassium (K⁺) were performed in the ten fluids where sufficient volume was available by flame photometry (EEL model 150 flame photometer) on cyst fluid diluted 1:400 in distilled water.

Histology of microcyst lining

The dissected microcysts were stained by haematoxylin and eosin (H & E) and periodic acid Schiff

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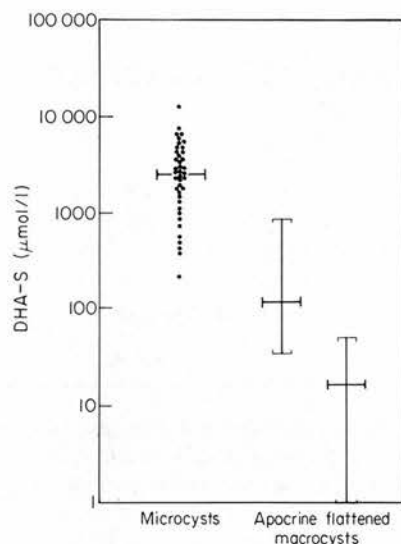


Fig. 1. Concentrations of DHA sulphate in 40 breast microcysts as assessed by radioimmunoassay. For comparison, the ranges and median values (horizontal bars) of DHA sulphate in apocrine and flattened macrocysts are also presented.

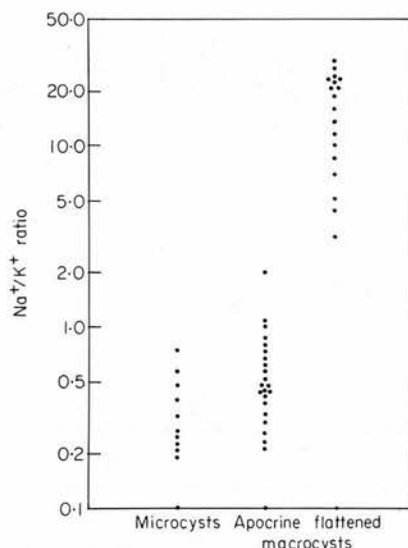


Fig. 2. Na^+/K^+ ratio in the ten microcyst fluids where sufficient fluid was available for these estimations. For comparison, the ranges of values for apocrine and flattened macrocysts are also presented.

technique after diastase digestion (PAS diastase). PAS diastase positive granules within cells was used as a positive method of identifying apocrine epithelium.²

Results

The concentration of DHA sulphate in fluids from 40 microcysts is shown in Fig. 1. For comparative purposes, the ranges and median concentrations of DHA sulphate in apocrine and flattened epithelial macrocysts are included. All microcyst fluids contained levels of DHA sulphate at least ten times greater than the upper limit of normal for plasma³ and the median value was almost 1000 times greater than that in plasma. Fluids from more than half the microcysts contained concentrations of DHA sulphate greater than that seen in any macrocysts. No microcyst had a DHA sulphate levels within the range of flattened epithelial macrocysts.

All ten microcyst fluids analysed for Na^+ and K^+ contained concentrations of K^+ in excess of those of Na^+ . The Na^+/K^+ ratio of these ten fluids is compared with that of apocrine and flattened macrocysts in Fig. 2. The Na^+/K^+ values for microcysts were all within the range seen in apocrine macrocysts.

All 40 microcysts were lined by epithelium which showed some apocrine features. The lining cells were frequently columnar and had apical snouts (Fig. 3) but, in others, the epithelium was cuboidal. The epithelium lining all microcysts contained PAS diastase positive granules at their luminal margin (Fig. 4).

Discussion

There exists two major populations of breast macrocysts which differ in both fluid composition and morphology of the lining epithelium.³⁻⁶ One population of cysts is lined by apocrine epithelium and contains fluid high in DHA sulphate and K^+ and the other is lined by flattened epithelium and has low concentrations of DHA sulphate and K^+ but high levels of Na^+ . Only a single population of their precursors has been identified in this study and these are lined by epithelium with apocrine features. In keeping with this, microcysts also contain fluid with high concentrations of DHA sulphate and K^+ . If all macrocysts are derived from this single population of microcysts, it remains to determine which factors determine whether a microcyst retains its active secretory lining when it enlarges into a palpable breast cyst.

Recent work has shown that patients with

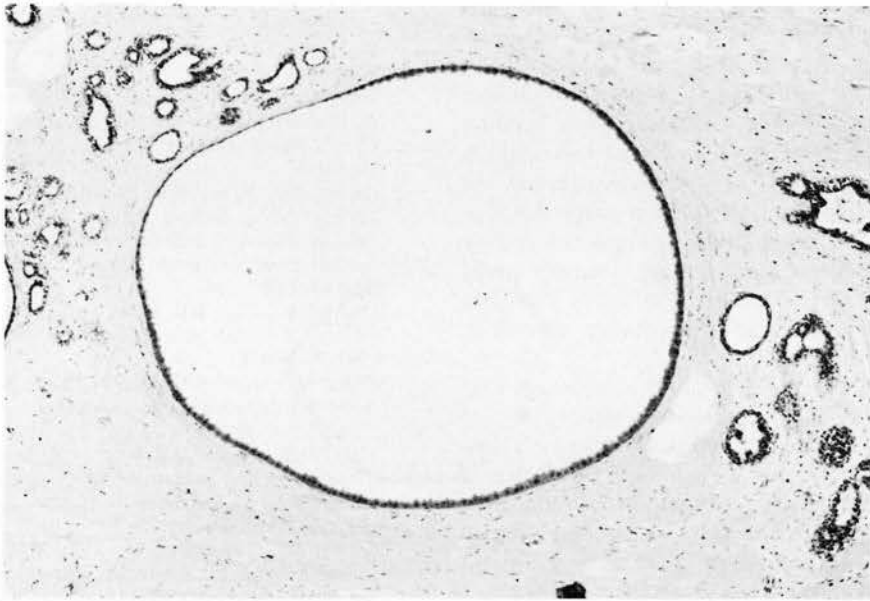


Fig. 3. Histology of a human breast microcyst. Note the columnar epithelium and the apical snouts (H & E $\times 40$).

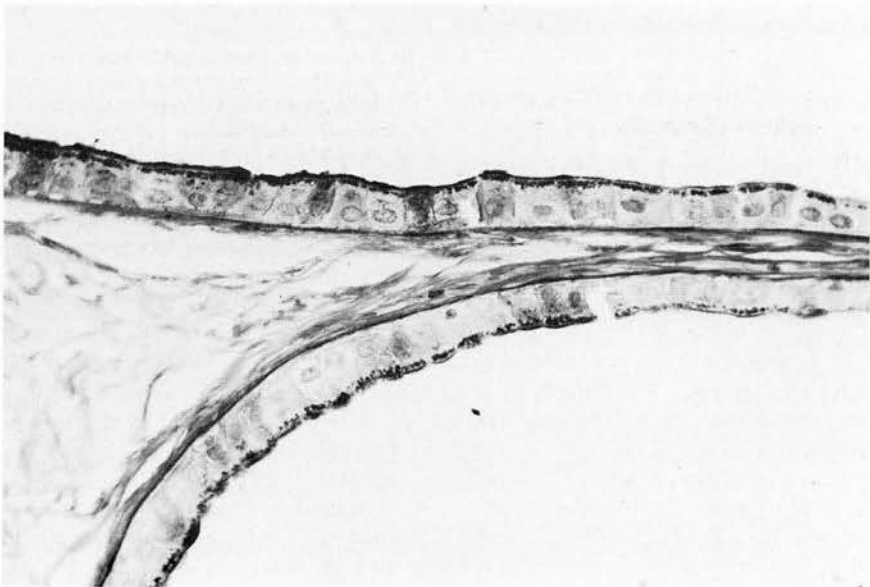


Fig. 4. Histology of the epithelium lining breast microcysts stained to show the glycolipid granules within the lining cells (PAS diastase $\times 200$).

apocrine cysts are more likely to (1) have multiple cysts,⁹ (2) develop further cysts⁹ and (3) be at greater risk of subsequent breast cancer.¹⁰ If there is only a single population of microcysts, as has been shown in the present study, then it may be that the same genetic or humoral factors which are

related to multiplicity and cancer risk¹⁰ determine whether a microcyst will develop into an apocrine or flattened macrocyst.

It has been considered that at least some breast cysts arise as a consequence of lobular dilatation and build-up of lobular secretions.¹¹ Studies of

breast secretions have shown that, although they contain high concentrations of DHA sulphate, Na^+ is the predominant cation, a composition different from both macro and microcysts. Simple blockage of the outflow of lobules with build-up of secretion resulting in microcyst formation is therefore unlikely. This study suggests that the important step in cyst formation is the development of epithelium with marked apocrine activity within the breast lobule. This is supported by the finding that in the early stages of microcyst formation, uniform apocrine change appears in the lobules involved.¹²

The finding of concentrations of androgen conjugates up to one thousand times higher than that in plasma shows that the hormonal micro environment of the breast is very different from that in plasma. The fact that the two differ so markedly may partly explain why studies of hormones in plasma and urine have failed to show consistent differences between controls and those with breast disease. Further studies are now required to determine whether measurements of hormones and their conjugates in breast fluids may provide a more direct link between hormones and diseases of the breast.

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SECTION E

MARKERS OF TUMOUR BEHAVIOUR

Oestrogen Receptor Activity and Endocrine Status in DMBA-Induced Rat Mammary Tumours

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Abstract—Oestrogen receptor activity (unfilled sites) has been determined in DMBA-induced rat mammary tumours and at sacrifice, the plasma concentrations of oestradiol-17 β and prolactin were determined by radioimmunoassay.

In intact animals, receptor levels were found to fluctuate in inverse relationship with the plasma oestrogen concentration, but following ovariectomy, levels were significantly reduced. Administration of oestrogen to ovariectomized animals did not detectably change receptor levels but administration of perphenazine significantly increased receptor levels towards those seen in intact animals.

In animals bearing two or more tumours, similar receptor concentrations were detected in tumours from the same animal.

It is concluded that the concentration of oestrogen receptor sites in rat mammary tumours is influenced by the circulating levels of both oestrogen and prolactin.

INTRODUCTION

DMBA-INDUCED rat mammary tumours, like some human breast cancers, are hormone-sensitive [1]. They contain receptors for oestrogen [2–6], prolactin [7, 8] and progesterone [9]. The oestrogen receptors in these rat tumours have been shown to be similar to those in human breast cancers chemically [10] and in their relation to response to endocrine surgery [4]. Prior to evaluating further the relationship between receptor measurements and the endocrine responsiveness of experimental mammary tumours, we have investigated the influence of endocrine status on the level of receptor activity.

MATERIAL AND METHODS

Tumours

Female Sprague–Dawley rats were given DMBA (30 mg) by intragastric instillation. Tumours induced within 6 months were measured with calipers twice weekly. Size was expressed as the product of measurements in two dimensions. On reaching 1.5 \times 1.5 cm tumours were either studied directly or subjected to endocrine manipulation prior to

receptor assay. Tumours were excised after exsanguination of the rat under ether anaesthesia. Vaginal smears were taken daily from 45 intact animals and the stage in the oestrous cycle was classified as described by Yoshinaga *et al.* [11].

Endocrine manipulation

Fourteen tumour-bearing rats were injected subcutaneously (s.c.) with perphenazine (5 mg/kg body weight) for 14 days.

A further 57 tumour-bearing rats were ovariectomized under ether anaesthesia and divided into three groups which were treated differently. In one group (OVX) of 11 rats, no further treatment was given until sacrifice 14 days later. In a second group (OVX + OE₂), 33 animals were left for 9–14 days and then injected s.c. with oestradiol 17- β (1–5 mg day in corn oil) for a further 6 days. In the third (OVX + Perphen) 13 animals were treated either immediately (9 rats) or after a period of 6–13 days of no treatment (4 rats) by subcutaneous injection of perphenazine (5 mg/kg body weight) for 6–14 days.

Multiple tumours in the same rat

In a total of 24 animals in various reproductive states (intact or endocrine manipulated),

oestrogen receptor activity was determined in two or more tumours from the same animal.

Determination of oestrogen receptor concentration

Tumour receptor concentrations were determined by a modification of a method described previously [12]. This modification was necessary to improve assay precision since by the original method, the low concentrations of receptor activity encountered in rat tumours lead frequently to non-linearity of the resulting Scatchard [13] graph.

Tumours were left in Tris buffer solution (0.25 M sucrose, 10 mM Tris and 1 mM ethylene diamine tetra-acetate pH 8.0) containing 0.5 mM dithiothreitol (14) for 1 hr at 4°C prior to homogenization at the rate of 300 mg/ml of Tris buffer and centrifugation of the homogenate at 105,000 *g* for 50 min (based on 15). The resulting cytosol was subjected to saturation analysis as previously described [12].

Determination of plasma hormone levels

Animals were exsanguinated at death through the abdominal aorta under ether anaesthesia, and the blood was used for the determination of plasma oestradiol-17 β and plasma prolactin concentrations [16]. Since the quantity of blood collected was sometimes limited, assay sensitivity for plasma oestradiol-17 β calculated for 4.0 ml plasma, was 0.13 ng/100 ml.

For plasma prolactin assay, ¹²⁵I-iodo-prolactin was prepared by the method of Redshaw and Lynch [17] a procedure which we found more reproducible than that previously employed. The sensitivity for 22 assays varied from 1 to 7.6 ng prolactin/ml with a mean value of 3.3 ng/ml. Inevitable deterioration of prolactin standard occurs on storage (NIAMDD instructions) and a correction for this has been made using the quality control data, a procedure which results in slightly lower values than those we reported previously.

Determination of cytosol protein concentrations

Protein concentration was determined by the method of Lowry, Rosebrough, Farr and Randall [18] using bovine serum albumin as standard.

Statistical analysis

Except where otherwise stated, statistical evaluation was carried out by the Wilcoxon rank test.

RESULTS

For most rat mammary tumours, the dissociation constant of binding (K_d) was found to be $0.4\text{--}0.5 \times 10^{-10}$ molar (see Table 1), in agreement with our earlier report [12].

The values found for tumour receptor concentration (P_0) by the method described in this paper are, however, 1.67 ± 0.29 (s.e., $N = 11$) times lower than those derived by the earlier method. By the modified method, a linear Scatchard plot was obtained for most of the tumours examined, and tumours with apparent receptor concentrations down to 0.05 fmoles/mg tissue clearly showed increasing displacement of (³H) oestradiol-17 β with increasing mass of non-radioactive oestradiol-17 β added.

Effect of the oestrous cycle on tumour receptor concentration

In 45 cycling rats, tumour receptor concentration varied during the oestrous cycle (Fig. 1 and Table 1) reaching a minimum in prooestrous which was significantly lower than the values in all other stages in the cycle ($P < 0.01$), and

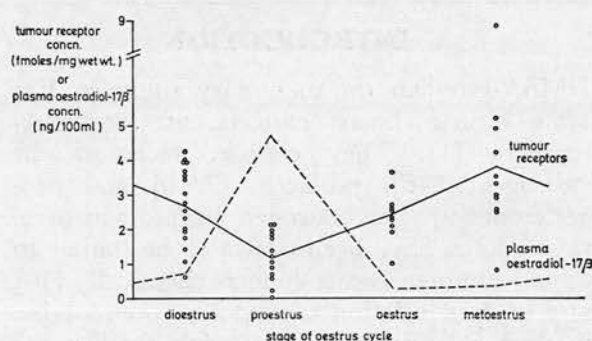


Fig. 1. The effect of stage in the oestrous cycle upon tumour oestrogen receptor concentration and plasma oestradiol-17 β concentration. Each circle represents the oestrogen receptor concentration in an individual tumour. Lines join the mean receptor concentration (—) and mean plasma oestradiol-17 β concentration (---). The standard errors for plasma oestradiol-17 β concentration have been omitted for clarity but are to be found in Table 1.

a maximum in metoestrus. The changes in receptor concentration were in inverse relation to those in plasma oestradiol-17 β (Fig. 1). The changes in plasma prolactin concentration followed, with a delay, those in oestradiol-17 β but did not reach statistically significant levels.

Effect of ovariectomy and oestrogen administration

Following ovariectomy, tumour receptor concentrations (Fig. 2) and the plasma levels of both prolactin and oestradiol-17 β (Table 1)

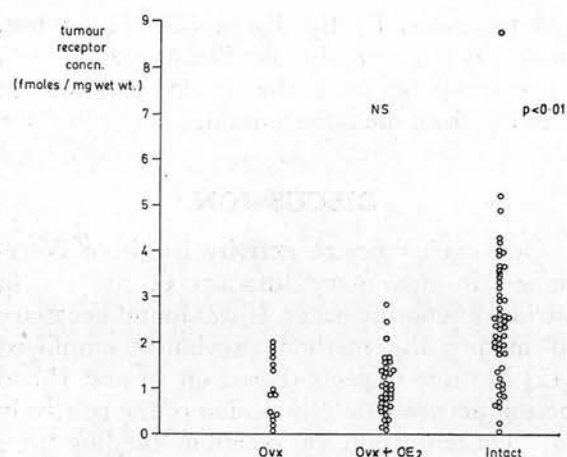


Fig. 2. The effects of ovariectomy and of ovariectomy followed by administration of oestradiol-17 β on tumour receptor activity. The values for the tumours from the intact animals shown in Fig. 1 are reproduced for comparison. Each circle represents the value for an individual tumour. *P* values refer to comparison with the concentrations in ovariectomised animals.

were significantly diminished relative to the values found in the tumours of intact animals ($P < 0.01$).

Administration of oestrogen to ovariectomized rats caused an insignificant increase in detectable receptor activity (Fig. 2), and a

restoration of prolactin levels to those seen in intact animals (Table 1).

Effect of perphenazine administration

Tumour concentrations of receptor activity in untreated rats were compared with the values in perphenazine-treated rats for both intact and ovariectomized states. The results are shown in Fig. 3 and Table 1.

In the intact animal, neither the mean tumour receptor concentration nor the plasma oestradiol-17 β concentration after perphenazine-treatment was significantly different from the value found in untreated animals in dioestrus. Although perphenazine-treatment significantly increased the plasma prolactin concentration over the value found in untreated rats in dioestrus ($P < 0.01$), the increased concentration was not significantly greater than the highest concentration observed in untreated animals at proestrus.

In ovariectomized animals, perphenazine-treatment caused a significant elevation in plasma prolactin concentration and in tumour receptor concentration ($P < 0.01$), whilst dissociation constant of oestrogen binding by the

Table 1. Tumour receptor concentrations and plasma hormone levels in tumour-bearing rats in various endocrine states

Reproductive state	No. of rats	No. of tumours	Tumour receptor activity			Plasma	
			K_d ($\times 10^{-10}$ M)	P_o (fmol/mg (tissue))	P_o , protein (fmol/mg (protein))	Oestradiol- 17 β (ng/100ml)	Prolactin (ng/ml)
			—	0.05	1	0.13	3
Intact proestrus	10	13	0.64 ± 0.10	1.21 ± 0.20	34 ± 7	4.70 ± 0.69	54 ± 16
oestrus	9	10	0.53 ± 0.04	2.42 \dagger ± 0.23	58 \ddagger ± 8	0.41 \dagger ± 0.15	51 ± 7
metoestrus	11	11	0.40 \S ± 0.03	3.77 \dagger ± 0.60	94 \ddagger ± 25	0.32 \dagger ± 0.07	35 ± 6
dioestrus	13	17	0.49 ± 0.05	2.70 \dagger ± 0.27	70 ± 11	0.70 \dagger ± 0.17	26 ± 6
Intact + per- phenazine (dioestrus)	14	19	0.39 ± 0.03	3.11 ± 0.30	72 ± 11	0.43 ± 0.08	77 \dagger ± 16
Ovariectomised OVX	11	15	0.72 ± 0.11	0.94 ± 0.17	23 ± 5	0.13 ± 0.02	12 ± 2
OVX + OE $_2$	33	36	0.62 ± 0.05	1.00 ± 0.11	22 ± 3	0.26 ± 0.05	40 \dagger ± 6
OVX + per- phenazine	13	16	0.42 \dagger ± 0.005	1.86 ± 0.19	44 ± 6	0.16 ± 0.03	59 \dagger ± 11

Results are the mean values ± 1 standard error.

*Sensitivity defined as described under "Methods and Materials" and first section of results.

$\dagger P < 0.01$, $\ddagger P < 0.02$, $\S P < 0.05$ when compared with appropriate reference value:

during oestrous cycle, values compared with that in proestrus; "Intact + perphenazine" values compared with "intact-dioestrus" value; "OVX + OE $_2$ " and "OVX + perphenazine" values compared with "OVX" value.

tumour was significantly reduced ($P < 0.01$) following perphenazine-treatment. No differences were noted between the values found for rats treated immediately after ovariectomy and those in which the start of treatment was delayed: accordingly the data for these two, slightly different modes of treatment have not been separated.

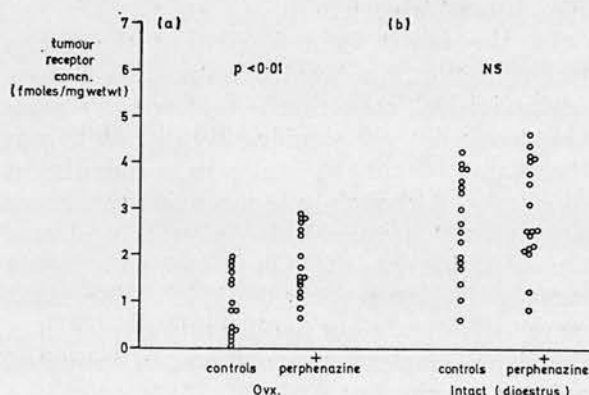


Fig. 3. The effect of perphenazine-administration in vivo upon tumour oestrogen receptor concentration in (a) ovariectomised and (b) intact rats. Each circle represents the value from an individual tumour. P values refer to the effect of perphenazine within each endocrine state.

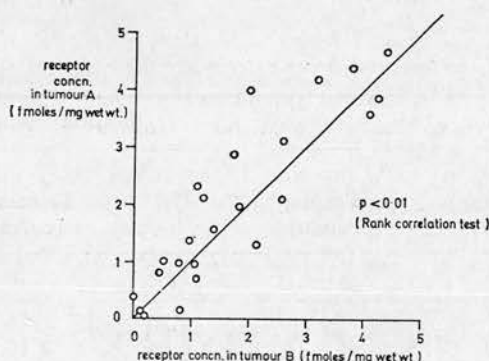


Fig. 4. Tumour receptor concentrations in rats bearing 2 tumours: receptor concentration for one tumour, A is plotted against that for the second tumour, B from the same rat. The rats were sacrificed in various reproductive states. Each circle represents the value from a single rat.

Receptor concentrations in multiple tumours from the same rat

When two or more tumours from the same animal were examined, the receptor concentration found for one tumour ("A") was more or less identical to the value for a second tumour ("B") from the same animal (Fig. 4). For animals with more than two tumours, only two values, selected by random selection (tables of random numbers), have been plotted.

In most cases, tumours from the same animal contained very similar concentrations of oestro-

gen receptors. By the Rank Correlation test, there was a highly significant ($P < 0.01$) correlation between the receptor levels in tumours from the same animal.

DISCUSSION

Oestrogen receptor activity has been determined in mammary tumours of the rat in various endocrine states. It was found necessary to modify the method previously employed [12] in three respects (based on 14 and 15) to permit accurate determination of the relatively low concentration of receptor binding sites present in these tissues. By this modified method, oestrogen receptor activity was detected in 98.5% of the 136 DMBA-induced mammary tumours examined. The receptor activity in these tumours was characterized by a dissociation constant of binding ranging from $0.16\text{--}1.80 \times 10^{-10}$ molar, and in most tumours dissociation constant was in the range $0.4\text{--}0.5 \times 10^{-10}$ molar.

The range of oestrogen receptor concentrations encountered in rat mammary tumours is approximately ten times lower than that found in human breast cancers [12]. A similar difference is seen between the concentrations of plasma oestradiol-17 β in the two species [16, 19].

Although most methods for determining oestrogen receptor concentrations detect only empty receptor sites, in general workers do not report the stage in the cycle at which tumours have been harvested for receptor assay or estimate plasma oestrogens at the time the tumour is harvested. We have shown that receptor concentrations fluctuate in inverse relationship with the prevailing oestradiol-17 β concentration, and that the tumour receptor concentration is a function of the stage in the oestrous cycle at which the tumour was excised.

This finding is in contrast to the recent work of Jordan and Jasper [20] who conducted receptor assays at 30°C, a temperature at which exchange between (^3H) oestradiol-17 β and endogenous oestrogen at filled receptor sites might occur. However, our results agree with those of Lee and Jacobson [21] and of Trams, Engel, Lehmann and Maass [22] in the rat and human uterus respectively, and of Maass, Engel, Nowakowski, Stolzenbach and Trams [23] in human breast cancer.

It is known that the effects of oestrogen upon the oestrogen receptor concentration within a tissue are complex: cytoplasmic receptor levels can be altered by increases in circulating oestrogen in two ways. In addition to (i)

filling empty receptors, which are then translocated to the cell nucleus, oestrogen may also (ii) stimulate the synthesis of new receptors [24, 25]. Our observations that tumour receptor levels firstly vary during the oestrous cycle and secondly, fall after ovariectomy, are, respectively, in keeping with these two effects.

In the rat, changes in circulating oestrogen level can also lead to alterations in the concentration of plasma prolactin (see e.g. [26].) This hormone has also been reported to influence oestrogen receptor levels in the rat mammary tumour [27–29]. In view of this, it is difficult to ascribe changes in receptor level to the effect of a single hormone. Nevertheless, in intact cycling animals, the tumour levels of detectable receptor activity found in this study were inversely related to changes in circulating oestradiol-17 β concentration and apparently unrelated to changes in plasma prolactin concentration.

In the ovariectomised rats treated with perphenazine, tumour receptor levels were clearly elevated in association with a significant increase in prolactin secretion, but in the absence of detectable oestrogen. This effect of prolactin on oestrogen receptor levels is in agreement with the findings of Leung and Sasaki [27, 28] and of Vignon and Rochefort [29]. Although perphenazine-treatment in ovariectomised rats also increased the affinity of the receptors for oestrogen (see Table 1), this apparent difference probably reflects the less accurate determination of K_d at low receptor levels rather than a specific effect of prolactin.

In ovariectomised rats treated with oestrogen, tumour receptor levels were not detectably changed relative to untreated controls, possibly due to the filling of empty sites by the injected oestrogen. The failure to detect any effect of perphenazine-treatment upon tumour receptor

levels in intact animals may either be due to the fact that the plasma prolactin concentrations found after treatment were not significantly greater than those found in untreated animals at proestrus and oestrus, or may indicate that the latter concentrations are already sufficient to maximally stimulate tumour receptor formation.

In animals bearing two or more tumours, despite the existence of a different clone of cells in each tumour, receptor levels were strongly correlated between tumours from the same animal, indicating that the endocrine milieu may be the major determinant of tumour receptor level.

These results support the view that endocrine status markedly influences tumour receptor level. In particular, we have indicated that oestrogen receptor levels may be increased by prolactin or reduced by the filling of empty receptor sites with oestrogen. If a similar situation obtains in women with breast cancer, then endocrine status at biopsy for receptor assay would influence the receptor levels detected and hence the predictive value of this parameter. The adoption of an exchange assay for measuring total cellular receptors should help by reducing the effect of circulating oestrogens on detectable receptor level for (i) clinical, predictive purposes and (ii) separating the effects of oestrogen and prolactin on receptor levels in the rat mammary tumour model.

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Endocrine manipulation and assessment of ovary-dependence

In order to assess ovary-dependence/independence, the tumours in one group of animals (group a) were measured at intervals with calipers before and after bilateral ovariectomy under ether anaesthesia. Tumour size was expressed as the product of measurements in two dimensions.

For a second group of animals (group b) tumours were measured before and during sequential manipulation by ovariectomy, no treatment for 10 days and oestradiol-17B administration for 6 days (1–5 µg/day s.c. in oil).

Ovary-independent tumours were defined as those tumours which continued to increase in size for the period of study following ovariectomy (usually by >30% over 9–14 days).

Ovary-dependent tumours were classified either as those tumours showing a continuous decrease in size for the period following ovariectomy (usually $\geq 50\%$ over 9–14 days)—group (a), or where animals were subsequently further treated by oestrogen administration, as those tumours regressing upon ovariectomy and clearly regrowing upon oestrogen administration—group (b).

Tumours showing extensive necrosis, changes due to a large cystic fluid content, or equivocal growth patterns were regarded as unassessable and were excluded.

Tumour receptor concentration in intact and ovariectomised rats bearing transplantable tumours

In an initial experiment, receptor concentrations were determined in the tumours from 23 intact, cycling animals (7 TG3, 10 TG5 and 6 TG6). A further 17 rats bearing 23 transplantable tumours (11 TG3, 11 TG5 and 1 TG6) were ovariectomised and left for 14 days prior to sacrifice and determination of tumour receptor concentration.

Comparison of receptor concentrations in transplantable tumours and DMBA-induced tumours

In a second study, receptor concentrations in 2 lines of transplantable tumours (TG3 and TG5) were compared with those in DMBA-induced tumours in each of 3 endocrine states.

For receptor assay, tumours were excised from;

- (a) 21 ovariectomised rats (6 regressing, DMBA-induced tumours and 15 growing, transplantable tumours, 8 TG3, 7 TG5);
- (b) 21 rats treated by ovariectomy and subsequent administration of oestradiol-17B (15 DMBA-induced tumours showing regression followed by regrowth, and 6 transplantable tumours showing continuous growth i.e. 1TG3, 5TG5);

- (c) 22 intact, cycling rats (10 DMBA-induced tumours, 12 transplantable tumours i.e. 10 TG3, 2 TG5).

Thus in groups (a) and (b), only animals showing clearly ovary-independent growth for a DMBA-induced tumour, or clearly ovary-independent growth for a transplantable tumour were examined. In group (c) the tumour specimen for assay was removed by biopsy (in oestrus, metoestrus or dioestrus, but not proestrus) under ether anaesthesia and the animals were then left approximately 14 days and ovariectomised. However, it was not possible to determine the ovary-dependence/independence of these tumours since the biopsy procedure alone frequently disturbed the growth of the tumour.

Measurement of plasma oestradiol-17B and prolactin concentrations

At sacrifice, the animals were bled through the abdominal aorta under ether anaesthesia. In all rats except group (c), bleeding preceded tumour excision, but in group (c), biopsy specimens of tumour were taken and animals were not sacrificed until approximately 4 weeks later.

Plasma was separated by centrifugation at 4°C and stored at –20°C for radioimmunoassay by methods previously described [7]. The sensitivities of these assays were calculated to be 3 ng prolactin/ml plasma and 0.13 ng oestradiol-17B/100 ml plasma when analyses were conducted on duplicate, 0.1 ml and single, 4.0 ml samples of plasma respectively.

Determination of oestrogen receptor concentration

Tumour tissue, removed either by biopsy under ether anaesthesia [group (c) only] or after killing by exsanguination, was placed in Tris buffer containing dithiothreitol (0.25 M sucrose 10 mM Tris, 1 mM ethylene diamine tetraacetate, 0.5 mM dithiothreitol, pH 8.0) and stored on ice for 1 hr. The concentration of oestrogen receptors in each tumour was measured by a method described previously [6].

Briefly, 600–800 mg tumour was trimmed free of adhering tissue and capsule at 4°C, cut up finely with scissors and homogenized in an ice-cooled tube at the rate of 300 mg/ml buffer, using a Silverson homogenizer (1 × 20 sec, 1 × 15 sec at maximum speed with a 45 sec interval for cooling between bursts). The resulting homogenate was centrifuged at $105,000 \times g$ for 50 min to yield a clear cytosol. Portions (0.1 ml) of cytosol were incubated, in duplicate, at 4°C with 10 pg [³H]oestradiol-17B (5060 counts/min) and varying quantities of non-radioactive oestradiol-17B (0, 10, 30, 50, 70, 90 and 20,000 pg) in a total volume of 1.2 ml Tris buffer (composition as

Table 1. Tumour receptor concentrations and plasma hormone levels in intact and ovariectomised rats bearing 3 lines of transplanted tumours

Tumour line	Reproductive state	No. of rats	No. of tumours	Tumour receptor activity			Plasma	
				K_d ($\times 10^{-10}$ M)	P_o (fmole/mg tissue)	P_o , protein (fmole/mg protein)	Oestradiol-17 β (ng/100 ml)	Prolactin (ng/ml)
				Sensitivity*				
				—	0.05	1	0.13	3
TG-3	Intact	7	7	0.46 ± 0.12	0.48 ± 0.15	11.5 ± 3.8	0.98 ± 0.52	28 ± 12
	OVX	7	11	0.36 ± 0.06	0.59 ± 0.10	11.3 ± 1.8	$0.12 \pm 0.02^\dagger$	$4.3 \pm 1.4^\dagger$
TG-5	Intact	10	10	0.54 ± 0.06	0.43 ± 0.11	14.5 ± 6.4	1.61 ± 1.75	32 ± 10
	OVX	9	11	0.43 ± 0.08	0.70 ± 0.16	16.0 ± 3.3	0.16 ($n=2$)	$5.4 \pm 1.1^\dagger$
TG-6	Intact	6	6	0.51 ($n=2$)	0.03 ± 0.02	0.7 ± 0.6	2.09 ± 1.51	39 ± 23
	OVX	1	1	—	0	0	0.01	53

Results are mean values ± 1 standard error. K_d =dissociation constant of oestradiol-17 β binding, P_o =concentration of oestrogen receptor sites. *Sensitivity defined as described under Materials and Methods. $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.10$ when OVX and intact values compared for same tumour line.

above except that dithiothreitol was omitted). After overnight equilibrium, free oestradiol-17B was removed by the addition of 0.5 ml charcoal suspension (0.15% charcoal, 0.0015% dextran in Tris buffer w/v), further incubation at 4°C for 15 min and centrifugation for 10 min at 4°C.

The protein-bound fraction was decanted into 5 ml organic scintillator solution for determination of ^3H activity. After correction for non-specific binding using the mean value for the 20,000 pg tubes, the data was analysed according to Scatchard [8] to derive the dissociation constant of binding (K_d) and the concentration of receptor sites (P_0). The latter was expressed both as fmole binding sites/mg wet tumour and as fmole binding sites/mg cytosol protein, using the protein concentration of the cytosol found on measurement by the method of Lowry, Rosebrough, Farr and Randall [9].

The sensitivity of the method was empirically defined as 0.05 fmole/mg wet tumour or 1 fmole/mg cytosol protein, since a clearly increasing displacement of [^3H] oestradiol-17B with increasing mass of non-radioactive oestradiol-17B present was generally observed above this receptor level.

Statistics

The significance of differences in receptor concentrations was evaluated by the Wilcoxon rank test.

RESULTS

Tumour receptor concentrations in intact rats

Low, but detectable levels of oestrogen receptor activity were found in two of the three lines of transplantable tumours, i.e. the TG3 and TG5 lines (Table 1). The dissociation constant of binding was of the order of 0.4×10^{-10} molar in each tumour line and no significant difference in receptor levels was apparent between the two tumour lines. In the third tumour line, (TG6), receptor activity was found in only 2 of 6 tumours examined and the levels detected were extremely low.

Effect of ovariectomy

The efficacy of ovariectomy was confirmed by the reduction of plasma oestradiol-17B concentrations to the level of the sensitivity of the assay (Table 1). Prolactin concentrations were also significantly reduced in 2 of the groups of rats bearing tumours (TG3 and TG5) and the apparent rise in prolactin level in the one rat bearing a TG6 tumour which was examined after ovariectomy is probably a spurious result (Table 1).

Despite these changes in plasma hormone concentrations following ovariectomy, no significant change in receptor level was observed in either TG3 or TG5 lines of tumours (Table 1, Fig. 2).

Comparison of receptor levels in transplantable tumours (ovary-independent) with levels in ovary-dependent, DMBA-induced tumours

Receptor levels in the transplantable tumours were compared with those in DMBA-induced tumours obtained from animals in each of three endocrine states. Firstly, 14 days after ovariectomy, (Fig. 3a), receptor concentrations were significantly ($P < 0.05$) higher in DMBA-induced tumours which regressed than in the tumours (non-regressed) of either TG3 or TG5 transplanted line, though there was a considerable overlap between groups. Secondly, when tumour-bearing animals were subjected to ovariectomy and subsequent administration of oestrogen (Fig. 3b), the receptor concentrations in ovary-dependent, DMBA-induced tumours were clearly higher than the concentrations in the ovary-independent, transplantable tumours ($P < 0.01$).

Thirdly, receptor concentrations in biopsy specimens from uncharacterised, DMBA-induced tumours were also significantly higher than the values found in transplantable tumours of either TG3 or TG5 line, though again there was a considerable overlap between the two types of tumour. Attempts to separate the biopsied, DMBA-induced tumours into ovary-dependent and independent types by their response to subsequent ovariectomy were unsuccessful: The biopsy procedure alone generally interfered with tumour growth.

DISCUSSION

Oestrogen receptor activity has been detected in low but significant amounts in 2 out of 3 lines of transplantable rat mammary tumours. The two lines containing low receptor levels maintained the morphological appearance of adenocarcinoma whilst the other tumour line without detectable activity showed some tendency to progress towards a sarcoma by the time of the study. Gala and Peck [10] have described two transplantable, mammary tumours, of which one was an adenoma with "high" levels of oestrogen receptor activity, whilst the other was a sarcoma devoid of receptor activity.

At the time of study, all three transplantable tumour lines exhibited ovary-independent growth. Prior to this work, however, at least one of these lines (TG3) had passed through an ovary-

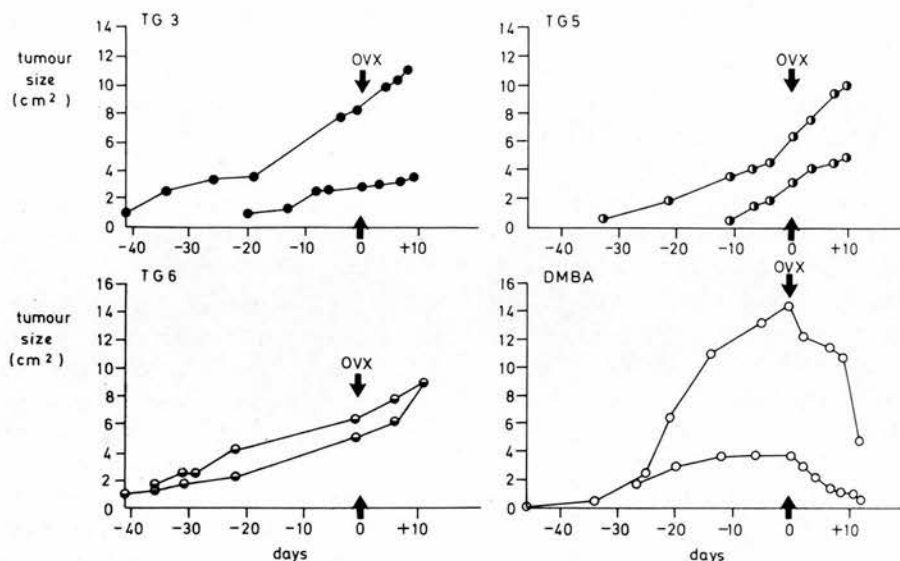


Fig. 1. The ovary-independent growth of 3 lines of transplantable mammary tumours. Tumour sizes were measured before and after bilateral ovariectomy of 2 rats bearing transplantable tumours, for each tumour line. For comparison, the effect of ovariectomy of the host upon the growth of two typical DMBA-induced tumours is also illustrated. OVX indicates the time of ovariectomy, and measurements are plotted against days pre (–) or post (+) ovariectomy.

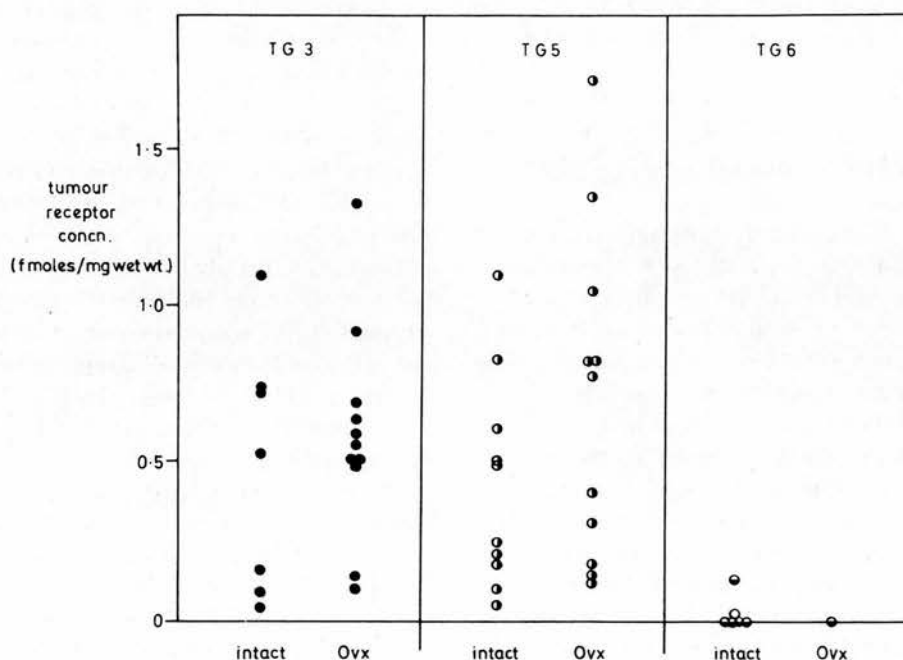


Fig. 2. Oestrogen receptor concentrations in each of 3 lines of transplantable mammary tumour. For each tumour line, receptor concentrations are shown in the tumours from intact and ovariectomised hosts. Each circle represents the value for a single tumour.

dependent phase in the very early generations. A similar progression from dependence to independence has been reported for certain strains of transplantable mouse tumour [11] and for other transplantable rat mammary tumours [12, 13].

The mere presence of oestrogen receptor activity in a tumour is thus not an indication of ovary-dependence, in agreement with the findings of others [11, 14–16] though as we have previously reported [17], the growth of at least some of these

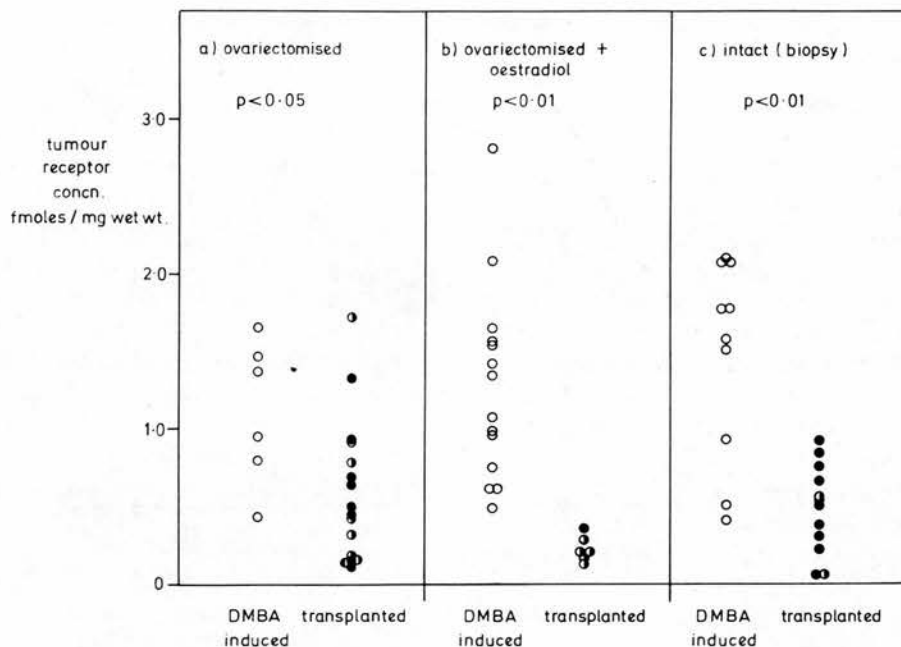


Fig. 3. Oestrogen receptor concentrations in ovary-independent, transplantable tumours were compared with values found in DMBA-induced tumours in each of 3 endocrine states. In (a), ovariectomised rats and (b), rats treated sequentially by ovariectomy and oestradiol administration, open circles denote receptor concentrations from ovary-dependent, DMBA-induced tumours; in (c), intact animals, open circles represent receptor concentrations in uncharacterised DMBA-induced tumours. Solid circles represent receptor concentrations in transplantable tumours of the TG3 line, and half-solid circles those in transplantable tumours of the TG5 line.

tumours is increased following oestrogen administration, i.e. is oestrogen-sensitive.

The R3230AC transplantable mammary tumour is also not ovary-dependent yet is sensitive to oestrogen [18] and similarly contains low levels of oestrogen receptor activity [14]. According to Sluysers and Van Nie [11] transplantable mammary tumours may change from "dependent" to "responsive" to "independent" tumours, in association with diminishing receptor levels upon successive transplantations. Although the data presented here was too small to show such significant differences in receptor levels between generations, it is our impression that the receptor levels in our TG3 and TG5 lines are also gradually diminishing with succeeding transplantations.

In the transplantable tumours studied here (Table 1), as in the R3230AC tumour [19] available receptor levels were undiminished by ovariectomy, though this finding could be the resultant of a decrease in total receptor sites and an increase in the number of available sites, upon removal of endogenous hormones. In contrast, the level of available receptor activity falls significantly after ovariectomy in most DMBA-induced tumours [6, 20, 21] where both receptor synthesis and general anabolism may depend on the presence of the ovary. This fall in available

receptor level in DMBA-induced tumours after ovariectomy, which we [6] and others (20, 21) have previously reported, was not seen in present work [Fig. 3, group (a) vs group (c)], probably because the receptor levels were measured in tumours from exsanguinated, ovariectomised rats (group a) but non-exsanguinated, intact rats (group c). The presence of a higher blood content in the latter type of tumour may account for our finding of receptor levels lower than those which we found previously [6] for DMBA-induced tumours from intact rats, and for the lack of difference between the values for tumours from intact and ovariectomised rats, since blood plasma can markedly suppress the level of receptor activity detected [22].

Early work with DMBA-induced mammary tumours implied very clear differences in magnitude of receptor levels between dependent and independent tumours of the rat [1-5]. More recently, Costlow, Buschow, Reichert and McGuire [23] also found clearly higher levels of both oestrogen and prolactin receptor activity in an ovary-dependent subline (MD) of a transplantable mammary tumour (MTW9) than in an ovary-independent subline (MA). In the present work, significantly lower mean receptor concentrations were found in the TG3 and TG5 tumour lines (ovary-independent) than in

DMBA-induced tumours (80–100% ovary-dependent) in each of three endocrine states. However, there was a considerable overlap in receptor values between the two types of tumour in intact animals, or in animals treated by ovariectomy only.

This finding of a less striking difference than was first proposed is in line with the views of Mobbs and Johnson [15], Boylan and Wittliff [16] and DeSombre, Kledzik, Marshall and Meites [13]. In the latter work, in particular, for both oestrogen receptors and prolactin receptors, there was a clear overlap between the concentrations found in dependent and independent mammary tumours. It is to be noted, however, that strictly only measurements on *biopsy* specimens taken prior to endocrine manipulation relate to the hormonal sensitivity at that time, and are comparable to the usual clinical situation where for prognostic use, biopsy material is taken *before* endocrine therapy.

For technical reasons (low incidence of DMBA-induced tumours in inbred rats, low transplant-acceptance rate in random-bred rats), the comparisons made here between DMBA-induced and transplantable tumours are complicated by the fact that the tumours were generated in rats of different strains. These strains of rat have different basal levels of prolactin (24, 25 and c.f. values in Table 1 with those in reference [6]), a hormone which has previously

been shown to modulate tumour receptor levels [20, 21]. Thus it is possible that the lower receptor levels detected in transplanted tumours may be related to the lower levels of this hormone which circulate in our inbred rats, rather than to a lesser degree of hormonal sensitivity. This possibility is currently being investigated.

Irrespective of the origin of this overlap in receptor concentrations between dependent and independent rat mammary tumours (e.g. effect of strain, hormonal levels or variation in degree of hormonal sensitivity) extrapolation of these findings to the clinical situation would suggest that predictions based on oestrogen receptor concentrations should be made with caution. Thus at the present time, only "receptor-negative" tumours, (i.e. those with receptor levels clearly below the overlap range) can be accurately predicted to fail to respond to endocrine therapy.

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Letter to the Editor

Progestogen and Oestrogen Receptor Activity in Ovary-Dependent and Ovary-Independent Tumours of the Rat

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ALTHOUGH mammary tumours containing oestrogen receptors are more likely to respond to endocrine therapy, the presence of the oestrogen receptor does not guarantee hormonal sensitivity [1, 2]: a critical, minimal concentration of receptors must be present [3-5]. Jensen has used the phrase "oestrogen receptor-rich" to distinguish such tumours from those lacking that concentration, "receptor-poor". An alternative approach has been suggested by McGuire and his colleagues [6], who noted that the synthesis of the progestogen receptor is oestrogen-dependent and thus the presence of progestogen receptor activity is a marker of a functional oestrogen receptor.

We have now examined three types of rat mammary tumour for both oestrogen and progestogen receptor activities: these are two lines of transplantable mammary tumour, TG-3 and TG-5, which are now ovary-independent [7] and dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumours which are predominantly ovary dependent.

Tumours were excised for use when 1.0-2.5 cm in diameter. Cytosol for receptor analysis was prepared as previously described [9], except that glycerol (10% v/v) and monothioglycerol (1% v/v) were included in the homogenisation buffer. Oestrogen receptor activity was determined using 100 µl portions of tumour cytosol as previously described [8]. Progestogen receptors were determined by in-

cubating overnight, on ice, either (a) 50-200 µl cytosol with 0.22 nM [7α - ^3H]Org-2058 (sp. act. 18.3 Ci/mmol) \pm 0.22-11.1 nM non-radioactive Org-2058 in Tris-glycerol buffer, or (b) 200 µl diluted cytosol (1.5 \times with buffer) with 0.5 nM [$6, 7$ - ^3H] R5020 (sp. act. 56.5 Ci/mmol) \pm 1-1000 nM non-radioactive R-5020 in Tris buffer. Free and bound hormone were separated by adsorption on charcoal [(a) 500 µl 0.15% w/v or (b) 400 µl 0.625% w/v]. The data were analysed according to Scatchard [9].

Sucrose density gradient analysis of the progestogen receptor was carried out as described for the oestrogen receptor [10] with minor modifications: glycerol (10% v/v) was included in the homogenisation buffer and gradients, and the [^3H]progestogen and the non-radioactive progestogen concentrations were, respectively, 8 and 32.2 nM for Org-2058 and 1.4 and 23.6 nM for R-5020.

A total of 31 tumours (17 DMBA-induced, 7 TG-3 transplanted and 7 TG-5 transplanted) were examined for progestogen receptor activity using [^3H]Org 2058 and for oestrogen receptor activity: in 15 tumours (5 of each type) progestogen receptor activity was also determined using [^3H]R-5020. The results are listed in Table 1. Oestrogen receptors were detected in all three types of tumour, the mean level detected being significantly higher in the DMBA-induced tumours than in either line of transplantable tumour ($P < 0.01$ by Wilcoxon Rank Sum test). The average dissociation constant of binding was 0.22×10^{-10} ($N = 31$, range $0.06-0.68 \times 10^{-10}$) molar. Progestogen receptor activity was detected in one of seven TG-5 transplantable tumours, in four of seven TG-3 transplantable tumours and in all seventeen DMBA-induced tumours when assays were carried out with

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Table 1. Oestrogen and progesterone receptor activities in three types of rat mammary tumour

Tumour type	Oestrogen receptor activity*		Progesterone receptor activity*			
	Fraction R +	fmole/mg tumour	Using [³ H]Org-2058		Using [³ H]R5020	
			Fraction R +	fmole/mg tumour	Fraction R +	fmole/mg tumour
	Sensitivity	0.05†	—	0.15‡	—	0.25§
TG-5 transplants	7/7	0.41 (0.11–0.66)	1/7	0.18 (0–0.18)	1/5	1.7 (0–1.7)
TG-3 transplants	7/7	0.46 (0.31–0.65)	4/7	0.36 (0–0.40)	4/5	2.1 (0–2.1)
DMBA-induced	17/17	3.24 (0.77–7.08)	17/17	26.9 (0.15–144.9)	5/5	40.6 (7–86)

*Fraction R + = No. tissues with detectable receptor activity/total tissues studied.

fmole/mg tumour = mean (and range) of concentrations found in R + tissues.

Sensitivities derived arbitrarily as follows:

† > 50 cpm displaced by addition of excess, nonradioactive steroid.

‡ > 150 cpm displaced by addition of excess, nonradioactive steroid.

§ > 200 cpm displaced by addition of excess, nonradioactive steroid.

[³H]Org-2058. There was generally good qualitative agreement between the results of the two assays for progesterone receptors using different ligands, and with [³H]R-5020, levels were detectable in one of five TG-5 transplanted tumours, four of five TG-3 transplanted tumours and all of five DMBA-induced tumours. The levels of activity were very low in all the transplanted tumours and in one (using Org-2058) or two (with R-5020) of the DMBA-induced tumours. Dissociation constant of binding was 1.7×10^{-10} molar (mean $n = 19$, range $0.25\text{--}6.3 \times 10^{-10}$) with the assay employing Org-2058, and 8.6×10^{-10} molar with that employing R-5020. Quantitatively, progesterone receptor levels were considerably higher in the DMBA-induced tumours than in the transplantable tumour lines ($P < 0.01$ by Wilcoxon Rank test).

The progesterone receptor extracted from DMBA-induced tumours was found to have a sedimentation constant of approximately 8–9s using either radioligand, but no peak in binding of [³H]progesterone could be demonstrated in the transplantable tumours. Amongst various compounds examined, only progesterone competed with [³H]Org-2058 for binding to cytosol from a DMBA-induced

tumour; the binding specificity of [³H]R-5020 is already well established [11, 12]. In our hands, these two progesterones were effectively equipotent (Relative Binding Affinities: R5020 96%, Org-2058 100% using [³H]Org-2058, and R5020 100%, Org-2058 100% using [³H]R5020). Neither progesterone bound detectably to cytosol, prepared from the prostate of a male rat and containing androgen receptors.

In our previous studies of oestrogen receptor activity [7, 8, 10], oestrogen translocation [13], steroid metabolism [14, 15] and sensitivity to hormones in culture [16] in these tumour types, we have not found such a striking difference as that seen here: whilst oestrogen receptor levels were seven times higher in the predominantly ovary-dependent, DMBA-induced tumours than in the ovary-independent transplantable tumours, progesterone receptor levels were 19–75 times higher. We conclude that these differences, seen between carcinogen-induced tumours and the advanced generations of transplantable tumours, provide further support for the hypothesis of McGuire *et al.* [6] that progesterone receptor activity may represent a useful index of hormonal sensitivity in breast cancers.

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Cellularity and the quantitation of estrogen receptors

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Keywords: cellularity, DNA, estrogen receptor, protein, wet weight

Abstract

Fifty estrogen receptor (ER)-positive breast cancers have been studied to determine the best way of correcting for differences in cellularity when expressing ER concentration. ER concentration expressed on wet weight and tumour cytosol protein bases showed a positive correlation with tumour cellularity. In contrast, ER concentrations expressed on a DNA basis were not significantly related to cellularity. Although such a mode of correcting for differences in cellularity was imperfect, it did yield a receptor concentration which was less dependent upon tissue cellularity and which may reflect more accurately the inherent receptor status of the tumour cells.

Introduction

It is now well established that the ER status of a breast cancer is of value in predicting the likelihood of response to endocrine therapy (1). Measuring the *amount* of ER present within a tumour improves the accuracy of this prediction of response (2, 3, 4).

The concentration of ER in a tumour depends on, amongst other factors, the number of malignant cells present (5, 6, 7). Thus the receptor concentration observed in a given tumour will be a complex measure of both the inherent receptor status of a tumour and its cellularity. The predictive value of ER quantitation may therefore be obscured by expressing results in a manner which does not take into account tumour cellularity.

In this study, we have investigated the inter-relationships between tumour cellularity and receptor concentration expressed on wet weight,

tumour cytosol protein, and DNA bases to determine which mode of expression provides the best compensation for differences in tumour cellularity and thus represents the best measurement of the amount of ER present per cell.

Material and methods

A total of 50 tumours from patients with primary breast cancer was studied. This group comprised 39 postmenopausal women and 11 premenopausal or menopausal women. In all cases the tissue obtained was from the primary tumour.

All tumours were assayed by the method of Hawkins et al. (8) but with the addition of monothioglycerol (1% v/v) and glycerol (10% v/v) to the homogenising buffer. Aliquots of tumour cytosol were assayed for cytosol protein concentration (9),

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and a portion of tumour adjacent to that used for ER assay was analysed for DNA content (10). From this data, each ER concentration was expressed as fmol/mg wet tissue, fmol/mg cytosol protein, and fmol/mg DNA. All the tumours included contained ER in concentrations greater than 0.25 fmol/mg wet tissue, 5 fmol/mg cytosol protein, and 100 fmol/mg DNA. Tumours with negative or borderline values for receptor concentration were excluded from this study.

A thin section of tumour adjacent to that used for receptor assay was placed in formol-saline and processed routinely for histology, including staining with haematoxylin and eosin. Cellularity was assessed visually by two independent observers using the system described by Roberts *et al.* (11) as modified by us previously (7). The relationship of the 'cellularity index' (ranging from 1–16) to other parameters was examined firstly by calculating the correlation coefficient (r) by the method of least squares, after logarithmic transformation of the data, and secondly by the Spearman rank correlation test. In view of the existence of an apparent 'outlier' in the data (the tumour with the lowest cellularity), all the data were analysed with ($n = 50$) and without ($n = 49$) the inclusion of this tumour. The correlation coefficients and ' p ' values shown are those derived from the method of least squares, except where analysis by the rank method yielded a significantly different result: in this case, the results of both analyses are included.

Results

(1) Relationship of tumour cellularity to DNA and protein contents

Tumour cellularity, assessed histologically as the 'cellularity index', was not related to the concentration of total protein measured in the cytosol ($r = +0.16$, $p > 0.2$, $n = 50$, or $r = +0.10$, $p > 0.4$, $n = 49$) but was significantly correlated with tumour DNA content ($r = +0.40$, $p < 0.005$, $n = 50$; $r = +0.33$, $p < 0.025$, $n = 49$), as shown in Fig. 1a and 1b, respectively. By the Spearman rank test, however, this correlation between cellularity and DNA con-

tent only attained significance when all the 50 tumours were considered ($r = +0.39$, $p < 0.05$ for $n = 50$ and $r = +0.24$, $p > 0.05$ for $n = 49$).

When the data from premenopausal and postmenopausal patients were analysed separately, insufficient data were available for a useful analysis in the former group. In the postmenopausal patients, however, again the same relationships were observed (i.e. cellularity index vs protein: $r = +0.07$, $p > 0.6$; cellularity index vs DNA content: $r = +0.44$, $p < 0.01$, $n = 39$).

(2) Relationship of receptor concentration to tumour cellularity

When expressed on a wet weight basis, receptor concentration was significantly correlated with tumour 'cellularity index' ($r = +0.40$, $p < 0.005$, $n = 50$ or $r = +0.42$, $p < 0.005$, $n = 49$, Fig. 2a) in agreement with our previous findings (5, 6, 7). Expression of receptor concentrations as fmol binding sites/mg total cytosol protein (Fig. 2b) did not eliminate this dependence of receptor concentration upon 'cellularity index' ($r = +0.34$, $p < 0.02$, $n = 50$ or $r = +0.38$, $p < 0.01$, $n = 49$). In contrast, receptor concentrations expressed as fmol of binding sites/mg DNA were no longer significantly related to the tumour 'cellularity index' ($r = +0.13$, $p > 0.30$, $n = 50$, or $r = +0.26$, $p > 0.05$, $n = 49$, Fig. 2c).

When the data from the postmenopausal patients were considered separately, the same pattern of relationships was apparent as was seen with the whole group (receptor concentration vs cellularity: wet weight basis $r = +0.46$, $p < 0.005$; protein basis $r = +0.44$, $p < 0.01$; DNA basis $r = +0.10$, $p > 0.5$; $n = 39$).

Discussion

These results demonstrate that there is a wide variation in both DNA (Fig. 1b) and estrogen receptor (Figs. 2a, b, c) contents in tumours which, by visual assessment of histological sections, contain similar numbers of malignant epithelial cells. The variation in receptor content was only slightly

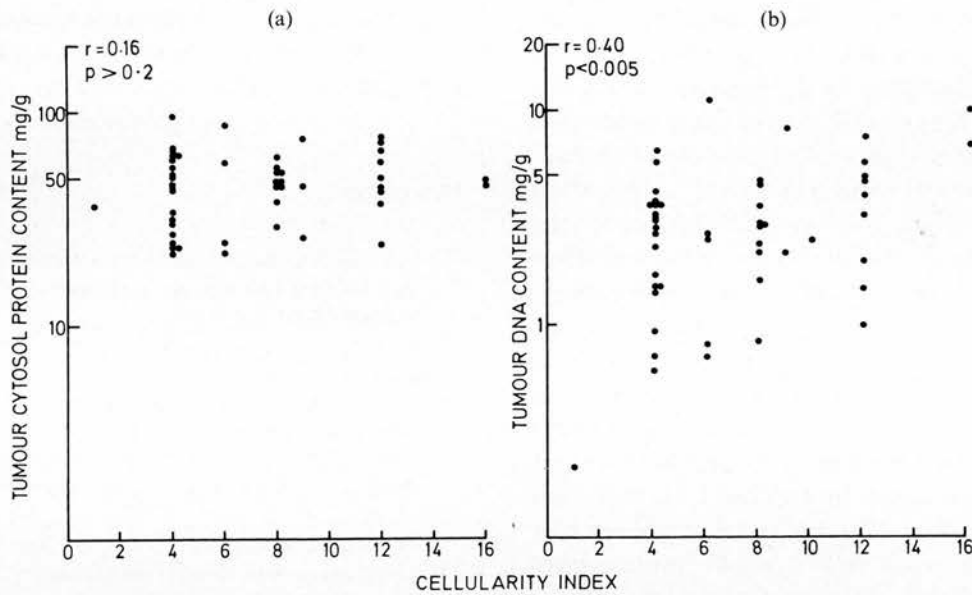


Fig. 1. The relationship of (a) tumour cytosol protein content, and (b) tumour DNA content, to tumour cellularity index. The correlation coefficients were (a) 0.16 ($p > 0.2$) and (b) 0.40 ($p < 0.005$) for all 50 tumours or (a) 0.10 ($p > 0.4$) and (b) 0.33 ($p < 0.025$) when the tumour of lowest cellularity was excluded.

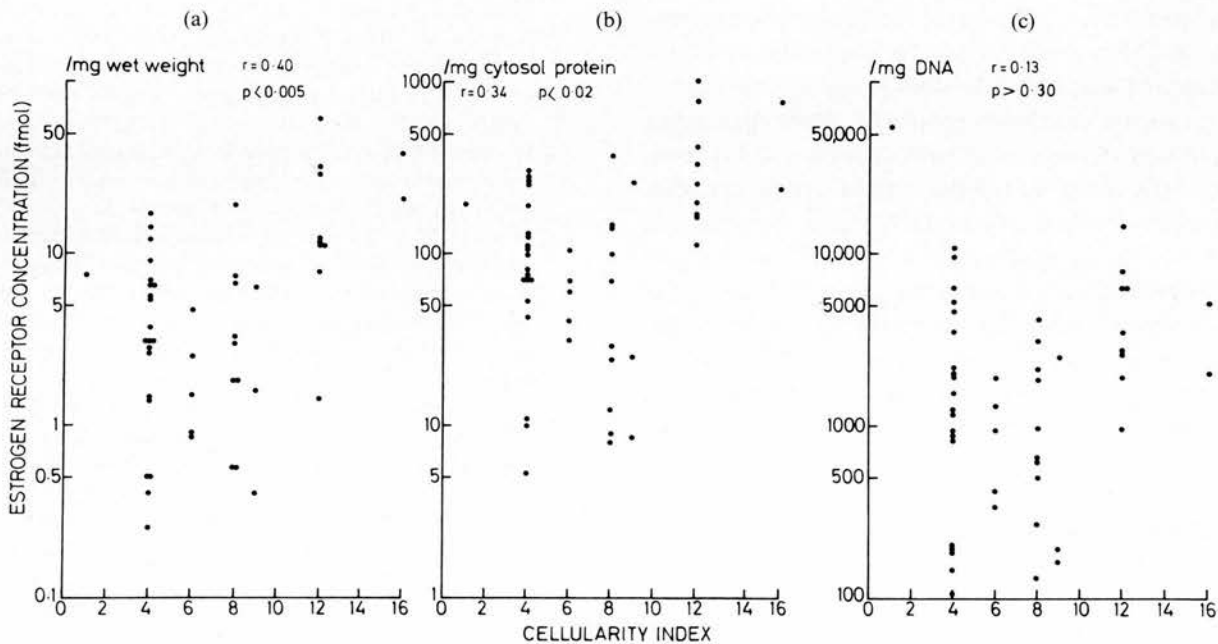


Fig. 2. The relationship of estrogen receptor concentration, expressed on the basis of (a) wet weight, (b) cytosolic protein, and (c) tumour DNA, to cellularity index. The correlation coefficients and probabilities, including ($n = 50$) and excluding ($n = 49$) the tumours of lowest cellularity index were respectively (a) $r = 0.40$, $P < 0.005$ and $r = 0.42$, $p < 0.005$, (b) $r = 0.34$, $p < 0.02$ and $r = 0.38$, $p < 0.01$, and (c) $r = 0.13$, $p > 0.3$ and $r = 0.26$, $p > 0.05$.

diminished when the 39 postmenopausal patients were considered on their own (not shown). Nevertheless, the concentration of receptors per mg wet tumour or per mg cytosol protein was dependent on the number of cells present whilst receptor concentration per mg tumour DNA was unrelated to cellularity. The scatter of results in the data for the latter may be partly due to the fact that DNA content was determined on an adjacent portion of tissue, rather than on the pellet from receptor assay.

The finding of a positive correlation between ER on a wet weight basis and tumour cellularity is in agreement with our earlier findings (5, 6, 7) and it implies that this method of expression does not allow for differences in cellularity. It has been suggested by others (12) that expression of receptor concentration on a total cytosolic protein basis should provide a correction for such differences. However, the results of the present work and another study (5) indicate that receptor concentrations/mg cytosol protein are still dependent upon the number of tumour cells present.

In this study, DNA content was a better biochemical assessment of cellularity than total cytosol protein. Thus expression of receptor concentrations on a DNA basis, whilst not eliminating intra-tumour variations (13), does provide some correction for differences in cellularity. Here the correction was incomplete in that there was still a tendency for the more cellular tumours to have higher receptor levels, even on a DNA basis. Nonetheless, such a mode of expression should yield a more accurate reflection of the inherent ER status of the tumour cells. Whether or not this mode of expression improves the value of tumour ER concentration in assessing prognosis or predicting response remains to be ascertained.

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Binding of [³H]-Methyltrienolone (R1881) by Human Breast Cancers

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Abstract—The synthetic radioligand [³H]-R1881 binds to both androgen and progesterone receptors; these two types of receptor activity can be separated by competition experiments with radioinert steroids of defined biological activity. Using two standard tissues, rat prostate and human uterus, which are sources of androgen and progesterone receptors respectively, the optimal conditions for the determination of each type of activity were established. For the purposes of routine assay, androgen receptors were quantified after saturation of progesterone receptor sites with 125 nM radioinert R5020 using [³H]-R1881 and increasing concentrations of radioinert R1881. Progesterone receptor activity could be identified using the same radioligand and competition with radioinert progesterone or R5020, though for routine purposes, progesterone receptors were quantified using the more specific radioligand, [³H]-R5020. The binding of [³H]-R1881 to tumour cytosol was examined in 122 human breast cancers. Seventy-two tumours (59%) showed binding. Androgen receptor activity alone was present in 16 tumours, progesterone receptor activity alone in 30 tumours and both types in 26 tumours. Tumours containing progesterone receptor activity also showed binding to the progesterone [³H]-R5020, whilst those containing androgen receptors alone did not. Androgen receptor concentration varied from 17 to 210 fmol binding sites/mg cytosol protein (mean value 68) and the mean K_d was 2.15×10^{-9} M. Progesterone receptor concentration varied from 25 to 1350 fmol binding sites/mg cytosol protein (mean value 410) and the mean K_d was 1.35×10^{-9} M. The biological significance of the presence of these types of receptor in human breast cancers is currently being assessed from clinical follow-up.

INTRODUCTION

THE SYNTHETIC androgen, methyltrienolone (R1881), forms a useful ligand for the measurement of androgen receptor activity. Unlike the natural androgen, 5 α -dihydrotestosterone, R1881 does not bind with high affinity to serum proteins such as sex hormone binding globulin and is not readily metabolised by target tissues [1]. This may be of particular value in human breast cancers which actively metabolise steroid hormones [2] and may be contaminated with serum-derived material. However, R1881 also binds to progesterone receptors [3, 4], which are often present in breast tumours [5]; this is a complicating factor in using the ligand for the measurement of androgen receptors in human breast cancer. The present study describes the characterization of the binding of tritiated R1881 in cytosol according to

whether competition occurs with androgen, progesterone or both types of steroid, the determination of the optimal conditions needed for the assay of each steroid receptor and the application of these methods to human breast cancers.

MATERIALS AND METHODS

Chemicals

Radioinert methyltrienolone (R1881), progesterone (R5020), [³H]-R1881 (58.2 Ci/mmol) and [³H]-R5020 (56.5 Ci/mmol) were gifts from Dr J. P. Raynaud (Roussel-Uclaf, France). Radioinert 5 α -dihydrotestosterone (5 α -DHT), progesterone and triamcinolone acetonide were obtained from Sigma (Poole, U.K.).

Tissues

Prostate was obtained from male Sprague Dawley rats 24 hr after bilateral orchidectomy.

Human uterus was obtained from patients undergoing hysterectomy.

Breast cancers were obtained at mastectomy or biopsy from patients with histologically proven disease.

All material was transported on ice to a cold room and immediately processed.

Cytosol preparation

Tissues were dissected free from surrounding fat and connective tissue, finely cut with scissors and homogenised at 0°C at a tissue: buffer ratio of 1:5 (w/v) in Tris buffer (10 mM Tris, 0.25 M sucrose and 1.5 mM EDTA to which 7.8 mg dithiothreitol, i.e. 0.5 mM, was added per 100 ml buffer immediately before use) employing a Silverson homogenizer at minimum speed for 3 × 15 sec with 1-min intervals for cooling. The homogenate was centrifuged at 105,000 g for 1 hr in an MSE Superspeed 50 centrifuge and the resulting supernatant was used as cytosol.

Binding measurements

All measurements were performed at 0°C. Cytosol (200 µl) was incubated with 500 µl Tris buffer, which contained competitor steroid where appropriate. Studies with [³H]-R1881 were performed in the absence and presence of 125 nM R5020. The only competitor studied in the absence of R5020 was progesterone at a final concentration of 10 nM, and those in the presence of R5020 were R1881 (final concentrations 1, 2, 4, 8 and 1000 nM) and 5α-DHT. For studies with [³H]-R5020, radioinert R5020 was used as competitor at final concentrations of 1, 2, 4, 8 and 1000 nM.

Tubes containing radioinert competitor and cytosol were left for 40 min before either [³H]-R1881 or [³H]-R5020 (final concentrations 0.5 nM) was added and the tubes mixed and incubated overnight. Bound steroid was then separated from free steroid by the addition of 400 µl dextran-coated charcoal (0.0625/0.625% w/v respectively). After 10 min the tubes were centrifuged and the total supernatant (bound fraction) was decanted into a counting vial

containing 10 ml of Liquifluor (Packard). The two phases were mixed, incubated at 37°C for 2 hr and the radioactivity measured in a Tricarb liquid scintillation counter.

Cytosol protein

The protein content of each cytosol was determined by the method of Bradford [6], using bovine serum albumin as standard.

RESULTS

Human uterus

All cytosols of human uterus showed binding of [³H]-R1881. Studies performed with radioinert steroids as competitors showed that R1881, R5020 and progesterone all inhibited the binding but that 5α-DHT was ineffective [Table 1(a)]. The effect of increasing concentrations of radioinert R5020 is shown in Fig. 1. Although binding was only completely inhibited with 500 nM R5020, only a trace of radioligand remained bound at concentrations above 100 nM. These results indicate that the binding was to progesterone receptors, a view supported by a high specific binding of [³H]-R5020 observed with the same cytosols (not shown).

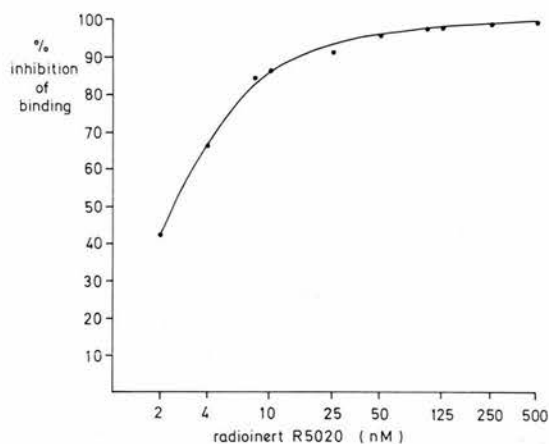


Fig. 1. The effects of increasing concentrations of radioinert R5020 on binding of [³H]-R1881 by human uterus. Percentage inhibition of binding was calculated as described in Table 1. Each point represents the mean of 3 separate estimations. Experimental conditions are as described in Materials and Methods.

Table 1. Characterization of [³H]-R1881 binding to cytosols from human uterus and rat prostate

Tissue	% inhibition of binding by competitor				
	5α-DHT (10 nM)	R1881 (10 nM)	Pg (10 nM)	R5020 (10 nM)	R5020 (125 nM)
(a) Human uterus	1.3 ± 0.7	57.7 ± 4.1	43.8 ± 4.0	86.2 ± 1.5	97.4 ± 0.5
(b) Rat prostate	71.2 ± 3.5	81.3 ± 3.6	5.7 ± 2.0	0	5.6 ± 3.2

Values represent the mean ± S.E. of the mean for 4 separate experiments. Percentage inhibition was calculated from the binding in the presence of the appropriate competitor and that in the absence of any competitor, both values being corrected for the non-specific binding found in the presence of 1000 nM radioinert R1881.

Rat prostate

All cytosols of rat prostate specifically bound [³H]-R1881. The binding as characterised by competition studies [Table 1(b)] was different from that in human uterus. In the rat prostate system radioinert R1881 and 5 α -DHT were effective competitors, whereas R5020 and progesterone had negligible effects. Only at concentrations in excess of 125 nM did R5020 consistently inhibit binding of [³H]-R1881. Data presented in Fig. 2 show the effect of including 125 nM R5020 on the binding of [³H]-R1881 analysed according to Scatchard [7]. At this concentration R5020 had little effect on the dissociation constant of binding (1.3×10^{-9} M) but reduced the concentration of receptor sites by about 5%. These results indicate that the binding of [³H]-R1881 in rat prostatic cytosol was to androgen receptors. Absence of progestogen receptors from rat prostate was confirmed by lack of specific binding of [³H]-R5020 in these cytosols (not shown).

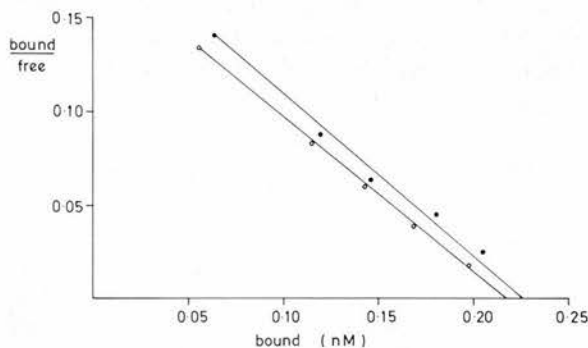


Fig. 2. Scatchard analysis of the binding of [³H]-R1881 in the absence (●) and presence (○) of 125 nM R5020 to rat prostatic cytosol. The latter was obtained by pooling prostates from 5 animals (protein concentration, 8.75 mg/ml). Each point represents the mean of triplicate estimations. Experimental conditions are as described in Materials and Methods.

Mixture of cytosols from human uterus and rat prostate

In order to determine whether it would be feasible to quantify androgen receptors in the presence of progestogen receptors by blocking the progestogen receptor with excess progestogen, experiments were performed with a mixture of cytosols from rat prostate and human uterus. Cytosols were prepared from rat prostate and human uterus as described in Materials and Methods and aliquots were mixed in equal volumes and assayed with tritiated R1881 and increasing concentrations of radioinert R1881 in the presence and absence of 125 nM R5020. In addition, the prostatic and uterine cytosols were each assayed separately after dilution with an equal volume of buffer. The results are shown in Fig. 3. Scatchard analysis showed that the binding

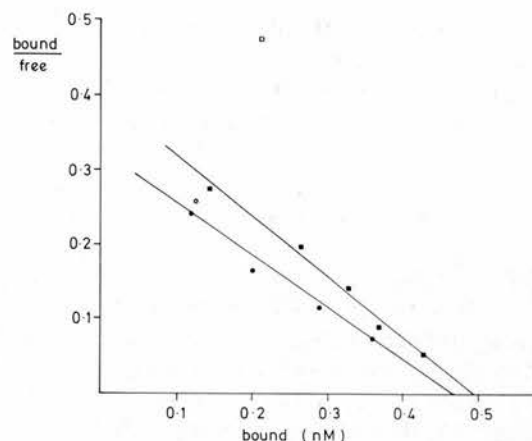


Fig. 3. Scatchard analysis of the binding of [³H]-R1881, in the presence of 125 nM R5020 to cytosol of rat prostate diluted with an equal volume of buffer (●), and to the same cytosol diluted with an equal volume of cytosol from human uterus (■). Open symbols represent the binding of each cytosol in the absence of both radioinert steroids R1881 and R5020. Each point is the mean of triplicate estimations. Prostatic cytosol was obtained by pooling prostates from 5 animals (protein concentration 9.5 mg/ml). The protein concentration of human uterine cytosol was 6 mg/ml. Experimental conditions are as described in Materials and Methods.

in the combined cytosol was 6.4% higher than that in rat prostatic cytosol alone, although the dissociation constants for the single and combined cytosols were similar. The additional binding in the combined cytosol over that seen in the prostatic cytosol alone may represent incompletely blocked progestogen receptor sites or the presence of small amounts of androgen receptor in the uterine preparation, though Scatchard analysis of the small amount of binding of [³H]-R1881 in the presence of 125 nM R5020 by human uterus failed to yield a satisfactory plot.

Human breast cancer

Binding of [³H]-R1881 was measured in the cytosols from 122 human breast cancers. Tumours were classified as showing specific binding if more than 200 cpm were displaced by the addition of 1000 nM radioinert R1881. This displacement is equivalent to 5 pmol/l (which, on the basis of an average tumour cytosol, would correspond to approximately 7 fmol/mg protein). Using this criterion, 72 tumours bound [³H]-R1881 (Table 2). The binding was characterised by using radioinert 5 α -DHT, progesterone and R5020 as competitors. These studies show that in 30 tumours, progesterone or R5020 but not 5 α -DHT blocked the binding (progestogen receptor activity); in 16 tumours 5 α -DHT but not progesterone or R5020 inhibited binding (androgen receptor activity); and in 26 tumours all 3 steroids acted as competitors (both progestogen and androgen receptor activities).

Table 2. Binding of [³H]-R1881 to cytosols of 122 human breast cancers

Binding	No. of tumours	(% of total)
None	50	(41)
Progestogen receptors*	30	(25)
Progestogen receptors + androgen receptors†	26	(21)
Androgen receptors‡	16	(13)

*Competition with progesterone and R5020.

†Competition with progesterone, R5020 and 5 α -dihydrotestosterone.‡Competition with 5 α -dihydrotestosterone.

Parallel studies were performed using [³H]-R5020 as radioligand. These showed that tumours which had bound [³H]-R1881 and in which binding had been blocked by progesterone and R5020 also specifically bound [³H]-R5020. Conversely, tumours which had failed to bind [³H]-R1881, or in which binding to [³H]-R1881 was displaced only by 5 α -DHT, failed to bind [³H]-R5020. Thus of the 122 tumours investigated, 56 (46%) contained progestogen receptors and 42 (34%) contained androgen receptors (Table 2).

Quantitation of androgen receptors was performed by measuring the effects of increasing levels of radioinert R1881 on the binding of [³H]-R1881 in the presence of 125 nM R5020. The concentrations of progestogen receptors in the same tumours were obtained by measuring binding of [³H]-R5020 in the presence of varying amounts of radioinert R5020. The results are shown in Table 3. Typical Scatchard plots for the binding of [³H]-R1881 by the cytosols from 2 human breast cancers (I and II), one of which also bound R5020 (II), are shown in Fig. 4. The mean dissociation constant of binding of [³H]-R1881 (in the presence of 125 nM R5020) for all the positive tumours was 2.15×10^{-9} M (range, 1.0 – 3.9×10^{-9} M) and that for [³H]-R5020 was 1.35×10^{-9} M (range, 0.4 – 3.2×10^{-9} M).

DISCUSSION

The use of [³H]-R1881 as a radioligand for the measurement of steroid receptors in human breast cancers suffers from the disadvantage that this synthetic steroid binds to both progestogen and androgen receptors. Since breast cancers may

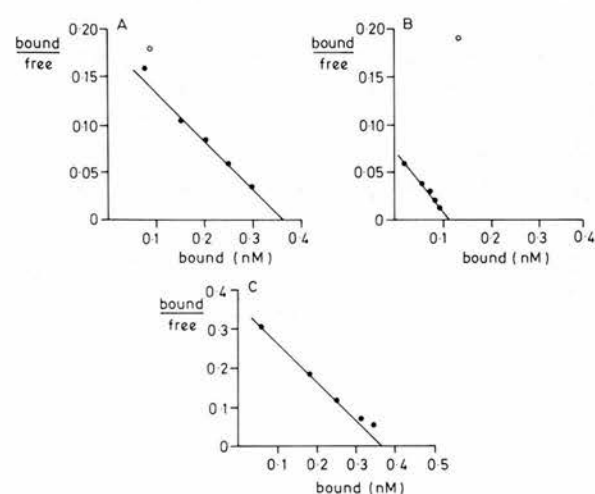


Fig. 4. (A) Scatchard plot for binding of [³H]-R1881 to cytosol of a human breast cancer (I) (protein concentration, 3.1 mg/ml). Solid symbols represent binding in the presence of a fixed concentration of radioinert R5020 (125 nM) and increasing concentrations of radioinert R1881; open symbol represents binding of [³H]-R1881 in the absence of both radioinert R1881 and R5020. This cytosol did not bind [³H]-R5020. (B) Scatchard plot for binding of [³H]-R1881 to cytosol of a human breast cancer (II) (protein concentration, 3.5 mg/ml). Symbols as for (A). This cytosol bound [³H]-R5020, shown in (C). (C) Scatchard plot for binding of [³H]-R5020 to cytosol of human breast cancer II. Each point represents the mean of duplicate estimations. (Assay conditions as described in Materials and Methods.)

contain one or both of these receptors, it is necessary to characterise the binding of [³H]-R1881 in each tumour. Therefore, in order to quantify receptor activity in tumours with both types of receptor, it is necessary to selectively block binding to one type of receptor while measuring

Table 3. Concentrations of androgen and progestogen receptor binding sites in cytosols of human breast cancers

Tumour type	Concentration of binding sites (mol/mg cytosol protein)	
	[³ H]-R1881 binding Mean \pm S.E.M. (range)	[³ H]-R5020 binding Mean \pm S.E.M. (range)
Androgen receptors alone	90 \pm 17 (19–217)	—
Progestogen receptors alone	—	428 \pm 61 (40–1345)
Androgen and progestogen receptors	55 \pm 9 (17–210)	370 \pm 73 (26–1250)
Total binding	68 \pm 9 (17–217)	413 \pm 46 (26–1345)

Table 4. Method for routine assay of androgen receptors in human breast cancer using [3 H]-R1881

All procedures to be performed at 0°C. 500 mg tumour homogenised in 2.5 ml Tris-sucrose buffer*

Homogenate centrifuged at 105,000 g for 1 hr. Resultant cytosol (200 μ l) added in duplicate to 500 μ l buffer containing 125 nM R5020† in the absence and presence of 1, 2, 4, 8 and 1000 nM R1881† (12 tubes)

Tubes mixed and left for 40 min. 100 μ l of [3 H]-R1881 (0.5 nM = 20,000 cpm) added, tubes mixed and left overnight

400 μ l of dextran-coated charcoal suspension added, tubes mixed, left for 10 min and centrifuged at 3000 rpm for 10 min. Supernatant decanted directly into scintillation vials containing liquifluor (10 ml), mixed and incubated at 37°C for 2 hr

Vials counted by liquid scintillation spectrometry and data analysed according to Scatchard using the 1000-nM tubes to correct for non-specific binding

*If additional material is available, by homogenising 750 mg in 3.75 ml buffer, it is possible to check that the binding of [3 H]-R1881 is to androgen receptor by also incubating 200 μ l of cytosol with 500 μ l of 5 α -DHT (10 nM) in the presence of R5020 (125 nM) in duplicate and to assess quantitatively progesterone receptor activity by incubating cytosol in duplicate with 500 μ l Tris-sucrose buffer with and without 10 nM progesterone (a further 6 tubes).

†Values refer to the final concentration in a total volume of 0.8 ml.

the concentration of the other. Such a technique has been used successfully to measure androgen receptors by suppressing binding to the progesterone receptor with triamcinolone acetonide [8]. However, under the assay conditions employed in the present study, excess of triamcinolone acetonide did not completely block progesterone receptor activity until very high concentrations, which also blocked androgen receptors, were used. In addition, since an assay for progesterone receptors using [3 H]-R5020 was already established in our laboratory, it seemed appropriate to measure androgen receptor activity with [3 H]-R1881 plus excess R5020 to block binding to the progesterone receptors.

In order to validate our method, we have studied the binding of [3 H]-R1881 in rat prostate, reported to be a source of androgen receptor in the absence of significant levels of progesterone receptor, and human uterus, reported to be a source of progesterone receptor in the absence of significant amounts of androgen receptor. Specific binding of [3 H]-R1881 to human uterus was readily blocked by progesterone and R5020, confirming that the binding in this tissue was predominantly to progesterone receptors. There was only a small but consistent (about 1%) inhibition of the binding by 5 α -DHT; this may represent the presence of trace amounts of

androgen receptors within the uterine cytosol. In contrast, the binding of [3 H]-R1881 by cytosol of rat prostate was effectively blocked by 5 α -DHT, whilst progestogens such as progesterone and R5020 had only minimal effects. The latter may indicate the presence of low levels of progesterone activity within rat prostate or a slight cross-reaction of progestogens with the androgen receptor.

R5020 at a concentration of 125 nM was selected for maximal blocking of progesterone receptor activity with minimal effects on androgen receptor activity. At this concentration R5020 blocked around 97% of the binding of [3 H]-R1881 to human uterus but only about 5% of that to rat prostate. Scatchard analysis of [3 H]-R1881 binding by rat prostate in the absence and presence of 125 nM R5020 showed similar kinetics, but with a reduction in receptor site concentration of around 5%. Experiments in which cytosols from rat prostate and human uterus were assayed separately and in combination confirmed that androgen receptors could be satisfactorily assayed in the presence of R5020, with only a very slight underestimation of receptor sites. Thus these results suggested that the binding of [3 H]-R1881 in these tissues and in human breast cancers could be characterised as being due to either progesterone receptors or androgen receptors, by including competitor studies with radioinert 5 α -DHT, R5020 and progesterone. It should be noted, however, that such assays for androgen receptor activity might have two sources of possible inaccuracy: (a) the excess concentration of R5020 used might block a small amount of androgen receptor and tumours possessing only low levels of androgen receptor might thus be misclassified as negative; (b) incomplete blocking of progesterone receptors by R5020 might lead to their being falsely classified as positive for androgen receptors, this being most likely to occur in tumours containing very high levels of progesterone receptors.

Specific binding of [3 H]-R1881 was detected in cytosols from 72 of 122 human breast cancers. Binding was characterised as being due to progesterone receptors (competition by progesterone and R5020 but not by 5 α -DHT) in 30, androgen receptor (competition by 5 α -DHT but not by progesterone or R5020) in 16, and to both androgen and progesterone in 26 tumours (competition by progesterone, R5020 and 5 α -DHT). This gives an overall incidence of androgen receptor activity of 34%. The corresponding figure quoted for other workers using different methods varies from 19 to 56% [9-13]. In this study the overall incidence for progesterone receptors was 46%. This figure is identical to that

which we found using [^3H]-R5020 as ligand in the same tumours, and is comparable to those reported by others [14, 15].

Quantitation of androgen receptors demonstrated a mean level in the receptor-positive tumours of 68 fmol/mg cytosol protein, with a mean K_d of 2.15×10^{-9} M. These values are comparable to those reported for the androgen receptor by others using different methods [8, 11, 13].

In conclusion, we have defined a method whereby the synthetic radioligand [^3H]-R1881 can be used for the simultaneous detection of progesterone and androgen receptors, though in a small number of tumours with very low levels of androgen receptor activity or very high levels of progesterone receptor activity the androgen

receptor status may be misclassified. Androgen receptor activity can be quantified by blocking the progesterone receptors with R5020. This method is summarised in Table 4. However, accurate quantitation of progesterone receptors is still best performed with the specific progesterone ligand, [^3H]-R5020. The clinical significance of the presence of androgen receptors, or of the simultaneous presence of both androgen and progesterone receptors, remains to be determined.

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Androgen Receptor Activity in Human Breast Cancer and its Relationship with Oestrogen and Progesterone Receptor Activity

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Abstract—Androgen receptor activity was measured in tumours from 122 patients with breast cancer. Forty-two tumours (34%) possessed androgen receptors at levels varying from 17 to 210 fmol/mg cytosol protein (mean value 68). No relationship was detected between androgen receptors and menopausal status of the patients and whether or not lymph nodes were invaded with tumour at the time of biopsy. There were, however, significant positive correlations between the presence of androgen receptors and that of oestrogen ($P < 0.05$) and of progesterone receptors ($P < 0.025$). These relationships suggest that androgen receptors may be of value in predicting the hormone responsiveness of breast tumours but definitive proof of this requires clinical follow-up of the patients studied.

INTRODUCTION

THE VALUE of oestrogen and progesterone receptors in predicting hormone responsiveness of human breast cancers is well established [1-4]. Androgen receptor activity in breast tumours has been less extensively investigated. This paper therefore describes the relationship of androgen receptors to other steroid receptors.

MATERIALS AND METHODS

Tumour was obtained from 122 patients with breast cancer. At the time of study 27 patients were premenopausal (experiencing regular menstrual periods), 90 were postmenopausal (at least 5 yr since their last regular menstrual period), four were menopausal (0-5 yr since the last menstrual period) and menopausal status was unknown in one patient. The breast cancer was obtained from the primary tumour at mastectomy (107 cases), from invaded lymph nodes during axillary clearance or sampling (13 cases) or from biopsy of local recurrent disease (two cases). The presence of malignant tumour was confirmed histologically in all samples. All material was transported on ice to a cold room and immediately processed.

The concentration of androgen receptors was determined by saturation analysis as previously described [5]. This involved incubation of a

tumour cytosol overnight at 0°C with [3 H]R1881 (58.2 Ci/mmol) in the presence of 125 nM R5020 and varying amounts of radioinert R1881.

Progesterone receptor assays were performed on the same cytosols as were used for the androgen receptor assay. The method involved incubating the cytosol overnight at 0°C with 1 nM [3 H]R5020 (56.5 Ci/mmol) in the absence and presence of 1, 2, 4, 8 and 100 nM R5020.

Oestrogen receptor measurements were performed in adjacent portions of the same tumours using a method previously described [6]. Tumour cytosol was incubated overnight at 4°C with [2, 4, 6, 7- 3 H] oestradiol-17 β and varying amounts of non-radioactive oestradiol-17 β .

In all receptor assays separation of free from bound steroid was by addition of dextran-coated charcoal: the bound fraction was measured by liquid scintillation counting. Concentration of receptors was determined by Scatchard analysis [7]. Activities were designated positive if in excess of 15 fmol/mg cytosol protein for androgen and progesterone receptors and greater than 5 fmol/mg cytosol protein for oestrogen receptors.

RESULTS

Androgen receptors were detected in 42 of 122 human breast cancers (34%). The incidence in primary tumours did not differ significantly from that in involved axillary lymph nodes (Table 1). Both of the local recurrences examined contained

androgen receptors. The concentration of androgen receptor measured varied from 17 to 210 fmol/mg cytosol protein (mean value 65 fmol/mg cytosol protein) and was similar in primary tumours and involved lymph nodes (data not shown).

The presence or absence of androgen receptors in relation to menopausal status and lymph node involvement (as assessed by histological examination of axillary nodes removed at the time of primary treatment) is shown in Table 2. No significant differences were apparent in either incidence or level of androgen receptors between the various subgroups.

There was, however, a significant positive correlation between the incidence of androgen and oestrogen receptors (Table 3). Thus 40% of oestrogen receptor-positive tumours also possessed androgen receptor activity, whereas only 19% of oestrogen receptor-negative tumours did so. No significant relationship was observed between levels of androgen and oestrogen receptors in tumours which contained both activities, but the mean level of androgen receptor was higher in oestrogen receptor-negative tumours than in those with oestrogen receptors (data not shown).

There was also a significant positive correlation between the incidence of the androgen and progesterone receptor (Table 4). Thus 46% of progesterone receptor-positive tumours showed

Table 3. The relationship between the incidences of androgen receptors (AR) and oestrogen receptor (ER) activity

Oestrogen receptor status	AR+	AR-	(% AR+)
ER+	36	55	(40)
ER-	6	25	(19)

The incidences of the two types of receptor were positively correlated: $\chi^2 = 4.18$, $P < 0.05$.

Table 4. The relationship between the incidences of androgen receptor (AR) and progesterone receptor (PgR) activity

Progesterone receptor status	AR+	AR-	(% AR+)
PgR+	27	32	(46)
PgR-	15	48	(24)

The incidences of the two types of receptor were positively correlated: $\chi^2 = 6.5$, $P < 0.025$.

androgen receptor activity compared with 24% of the progesterone receptor-negative group. Levels of the two types of receptors were not significantly related (data not shown).

The tumours could be subdivided into eight different groups depending on the presence/absence of the three types of receptors measured (Table 5). Each combination of receptors was

Table 1. Incidence of androgen receptor activity in human breast cancer

	No. of tumours with androgen receptors†	Total	(% positive)
Primary	35	107	(33)
Lymph node	5	13	(42)
Local recurrence	2	2	(100)

†Androgen receptor positive, >15 fmol/mg cytosol protein.

Table 2. Effects of menopausal status and lymph node involvement on incidence of androgen receptor activity

	No. of tumours with androgen receptors†	Total	(% positive)
Menopausal status			
premenopausal	6	27	(22)
menopausal	1	4	(25)
postmenopausal	34	90	(38)
Lymph node status			
involved	21	70	(30)
not involved	18	46	(39)

Differences between groups not significant by the chi square test.

Table 5. Inter-relationships between androgen receptor, oestrogen receptor and progesterone receptor activity in human breast cancers

			No.	(% of total)
AR+	ER+	PgR+	27	(22)
AR+	ER+	PgR-	9	(7)
AR+	ER-	PgR-	6	(5)
AR-	ER+	PgR+	32	(26)
AR-	ER+	PgR-	23	(19)
AR-	ER-	PgR-	25	(21)
Total			122	(100)

represented except that no oestrogen receptor-negative, progesterone receptor-positive tumours were identified. Only six tumours contained androgen receptors alone whereas 27 had all three types of steroid receptor.

DISCUSSION

The role of both oestrogen receptors and progesterone receptors in hormone-responsive human breast cancer has been extensively evaluated. In contrast, the significance of androgen receptors in breast tumours is still uncertain. Response to certain forms of endocrine therapy is more likely in androgen receptor-positive rather than androgen receptor-negative human breast cancers [8-10], but the numbers of patients studied are small and results remain to be confirmed in larger investigations.

Although the determinations reported here have largely been performed on primary tumours from patients with early breast cancer (and therefore data on response to endocrine therapy is as yet minimal), these measurements provided the opportunity to investigate in a relatively large series the interrelationships of androgen receptors with other steroid receptors of established value.

The incidence of androgen receptors in human breast cancer of 34% and the mean level of 68 fmol/mg cytosol protein as detected in the present study are similar to the values reported by others using different methods [9, 10, 11-13]. Incidence and level were not significantly influenced by the site of tumour biopsy, menopausal status or lymph node status of the

patients. A similar lack of association between these factors has been reported by others [14], although, as in the present study, there was a tendency for a higher incidence of androgen receptor activity to be observed in tumours from postmenopausal patients.

In the present study there was a significant, positive correlation between the presence of androgen receptors and that of oestrogen receptors. Others have reported a similar correlation [10, 14, 15], although it is important to note that the correlation is not absolute and that many tumours contain only one of the receptors [16].

The presence of androgen receptors also correlated with that of progesterone receptors, though, as with oestrogen receptors, there was a substantial number of tumours which contained either type of receptor alone. Because of the positive correlations between androgen receptors and both oestrogen and progesterone receptors, the presence of androgen receptors within breast cancers may also reflect hormone-dependence. However, the presence of androgen receptors in about 20% of oestrogen receptor-negative tumours, which classically are unlikely to respond to endocrine therapy, argues against androgen receptors alone being of predictive value. Nevertheless, the simultaneous presence of androgen receptors along with oestrogen and progesterone receptors (in 22% of tumours in this study) might increase the likelihood of response over that associated with tumours possessing oestrogen and progesterone receptors alone. Equally, others have suggested [9, 10] that the presence of androgen receptors might predict particularly for specific forms of endocrine therapy, especially those which affect tumour androgen levels. Clearly, clinical follow-up of the patients in this study is required to elucidate these possibilities.

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Prostaglandins in human mammary cancer

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Summary Prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$) were measured by Gas Liquid Chromatography - Mass Spectrometry (GLC-MS) in extracts of 100 human mammary carcinomas. All tumours contained measurable amounts of both prostaglandins but wide variations between individual tumours were observed. Values for PGE_2 ranged from 7 to 762 ng g^{-1} tissue with a median of 100 ng g^{-1} tissue. Values for $PGF_{2\alpha}$ ranged from 3 to 475 ng g^{-1} tissue (median 60 ng g^{-1} tissue). There was a highly significant positive correlation between amounts of the 2 prostaglandins in individual tumours. Amounts of both PGE_2 and $PGF_{2\alpha}$ were not significantly related to the menopausal status of the patients or the presence of oestrogen and progesterone receptors.

Human mammary cancers appear to produce prostaglandin-like material, and this may be involved in tumour growth and metastases (Bennet *et al.*, 1977; Rolland *et al.*, 1980). Most investigations have, however, employed bioassay or radioimmunoassay techniques which do not definitively identify prostaglandins. A single study has reported on determinations performed by GLC-mass spectrometry but the number of tumours examined was relatively small (Stamford, 1983). The present investigation represents a study of 100 human mammary cancers in which PGE_2 and $PGF_{2\alpha}$ have been identified and quantitated by GLC-mass spectrometry. Values of prostaglandins have then been related to tumour steroid receptor status.

Materials and methods

Tumours

Tumour was obtained from 100 women with carcinoma of the breast. These patients comprised 15 premenopausal, 10 perimenopausal (within 5 years of the last menstrual period) and 75 post menopausal women. Tumour was removed at mastectomy or biopsy from the primary cancer (in 88 women), by biopsy of invaded lymph node (in 9 cases) or secondary recurrences (in 3 cases). For comparative purposes, material was also obtained from benign fibroadenoma of the breast in 5 women and from histologically normal breast tissue in 3 women. This material was placed on ice and

immediately transferred to the laboratory. Following removal of tissue for histopathological diagnosis, the remaining material was dissected free of extraneous fat and divided for prostaglandin and steroid receptor assays.

Measurement of prostaglandins

Formation of derivatives Tumour samples were weighed and homogenized in ethanol (2.5 ml). To 1 ml duplicates of each sample were added 20 ng of the internal standards, (20-ethyl $PGF_{2\alpha}$ and 20-methyl PGE_2). Oximes were formed by adding hydroxylaminehydrochloride solution (50 mg ml^{-1}) in sodium acetate buffer (3 mol l^{-1} , pH 5.2) and heating for 30 min at 60°C . The samples were extracted and purified using a C18 Seppak column, washed with 10 ml iso-octane and then 10 ml 50% methanol to remove neutral lipids. The prostaglandins were then eluted with 90% methanol (10 ml). This fraction was evaporated to dryness and the residue dissolved in ethyl acetate:ethanol (1:1 v/v), transferred to a small flat-bottomed tube and methylated with 100 μl diazomethane solution. Excess diazomethane and solvent were then evaporated. The residue was further derivatized to the t-butyldimethylsilyl ether by adding 65 μl 2M-t-butyldimethylchlorosilane and 65 μl 4M-imidazole (both in dimethylformamide). After mixing, this solution was transferred to a narrow glass tube which was then sealed and heated for 30 min at 130°C . The excess reagents were removed from this mixture by eluting the derivative with 3 ml hexane:ethylacetate (3:1 v/v) from a Sephadex LH-20 column. The solvent was evaporated and 20 μl toluene added to the derivative. About one twentieth of this mixture was injected into the gas chromatograph.

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Gas chromatography – mass spectrometry
Samples were analysed with a Erba Science gas chromatograph coupled through an all-glass jet separator to a V.G.305 mass spectrometer. An open tubular column coated with SE₃₀ and 12 m long (SGE Ltd. London) was used. The flow rate of helium was 5 ml min⁻¹. The temperatures were maintained as follows: gas-chromatography column 280°C; separator and lines 260°C; source block 270°C. The signal was processed by a 2150 data system to allow separate GC traces for each.

For analysis of PGF_{2α} the mass used was m/z 653 and 681 and for PGE₂, m/z 666 and 680. The ions measured were the M-57 ions resulting from the loss of a t-butyl radical from the molecular ion. Quantitation was achieved by comparing the areas of the sample peak with those of the corresponding standards. Procedural losses were corrected by monitoring the recovery of the internal standards, 20 methyl PGE₂ (m/z 680) and 20 ethyl PGF_{2α} (m/z 681). The intra-assay precision was 13%; values for interassay precision were 18 and 21% for PGF_{2α} and PGE₂ respectively.

Oestrogen receptors

Concentration of oestrogen receptors was determined by saturation analysis (Hawkins *et al.*, 1975). Tumour cytosol was incubated overnight at 4°C with [³H] 17 β-oestradiol. Separation of free and bound steroid was by addition of dextran-coated charcoal; the bound fraction was measured by liquid scintillation counting. Concentration of receptors was determined by Scatchard analysis (Scatchard, 1949). Activities in excess of 5 fmol mg⁻¹ cytosol protein were designated receptor positive.

Progestogen receptors

Cytosol was incubated with a fixed concentration of [³H] Organon-2058 (0.22 nM) and varying concentrations of non-radioactive Organon-2058 (0.22–11.1 nM) with overnight binding at 0°C and separation of free and bound hormone by charcoal adsorption (Hawkins *et al.*, 1981). Activities in excess of 10 fmol mg⁻¹ cytosol protein were designated receptor positive.

Protein concentration in the cytosol was determined by the method of Bradford (1976).

Statistical analysis

Non-parametric tests (i.e. Wilcoxon's Rank Test and Spearman's Rank correlation) were used throughout these studies.

Results

Measurable amounts of PGE₂ and PGF_{2α} were

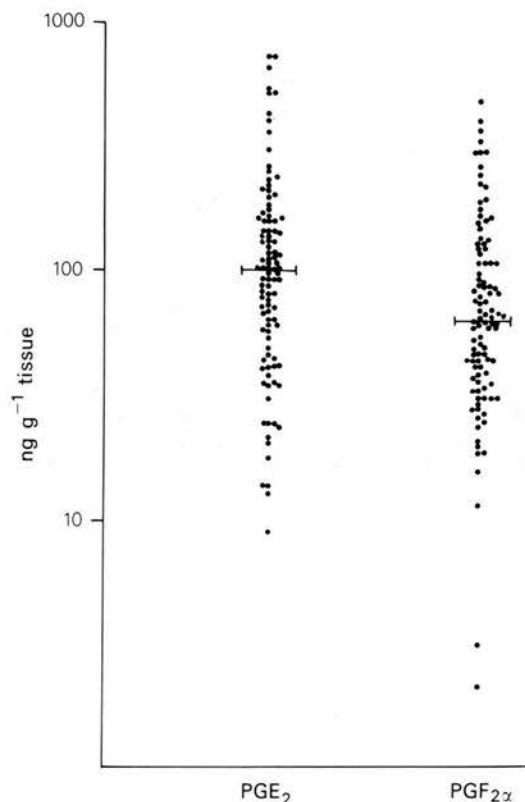


Figure 1 Levels of PGE₂ and PGF_{2α} in 100 human breast cancers. Lines represent the median values.

detected in all carcinomas. Levels in individual tumours are shown in Figure 1. Range of values for PGE₂ was from 7 to 762 ng g⁻¹ tissue with a median value of 100. The corresponding range for PGF_{2α} in the same samples was from 3 to 475 ng g⁻¹ tissue (median 60). Values in a group of 5 fibroadenomas ranged from 2–19 ng g⁻¹ tissue (median 9) for PGE₂ and 6–14 ng g⁻¹ tissue (median 12) for PGF_{2α} and those for 3 specimens of histologically normal breast were 7–14 ng g⁻¹ tissue for PGE₂ and 5–18 ng g⁻¹ for PGF_{2α}.

Within the group of breast cancers, there was a highly significant correlation between amounts of PGE₂ and PGF_{2α} (Figure 2). (Spearman's Rank correlation coefficient = 0.543 *P* < 0.001).

There was no significant difference between prostaglandin levels in primary tumours, lymph nodes and secondary recurrences (Data not shown). Nor were there significant differences in amounts of either PGE₂ or PGF_{2α} in pre, peri and postmenopausal patients (Figure 3).

Oestrogen receptor activity was detected in 68 tumours (68%) and the relationship between the presence of receptors and prostaglandin content is

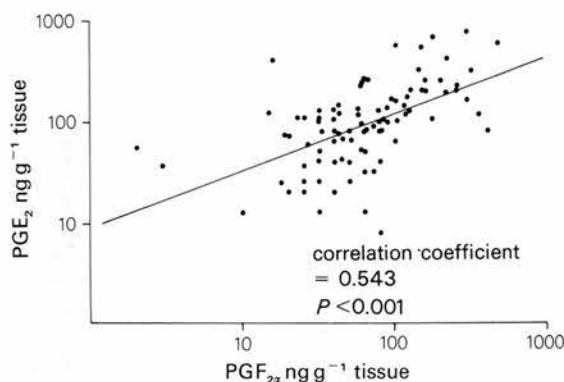


Figure 2 The relationship between levels of PGE_2 and $\text{PGF}_{2\alpha}$. Line is that of linear correlation. Correlation coefficient by Spearman Rank test 0.543 $P < 0.001$.

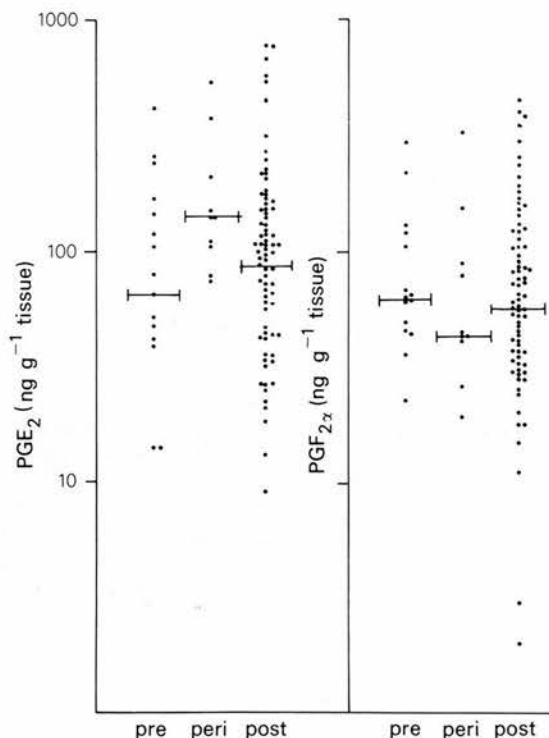


Figure 3 Levels of prostaglandins in tumours from pre, peri and postmenopausal patients. Lines represent median values. No significant differences between the groups by Wilcoxon Rank Test.

shown in Figure 4. The median value for both PGE_2 and $\text{PGF}_{2\alpha}$ was higher in tumours with oestrogen receptors as compared to those without receptors, but the difference between the two

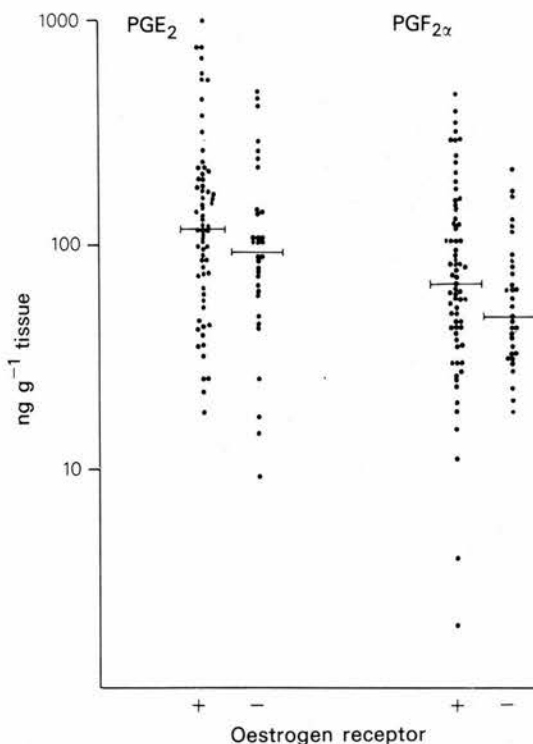


Figure 4 Levels of prostaglandins in oestrogen receptor positive (+) and negative (-) tumours. Lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.

groups failed to reach statistical significance. ($P < 0.1$ and $P < 0.2$, respectively by Wilcoxon Rank Sum Test).

Progesterone receptors were detected in 34 of the 93 tumours investigated (37%). There was no significant difference between either PGE_2 or $\text{PGF}_{2\alpha}$ levels in progesterone receptor positive and negative tumours (Figure 5). ($P < 0.8$ and $P < 0.9$ respectively by Wilcoxon Rank Sum Test).

Discussion

To date, the evidence that human breast cancers contain significant amounts of prostaglandins has been based on data from either bioassay or radioimmunoassay. These techniques, although of value, do not accurately identify different prostaglandins. In the present study, therefore, we have used the more definitive technique of GLC-mass spectrometry which has previously only been employed for prostaglandin measurements in a small group of breast cancers (Stamford, 1983).

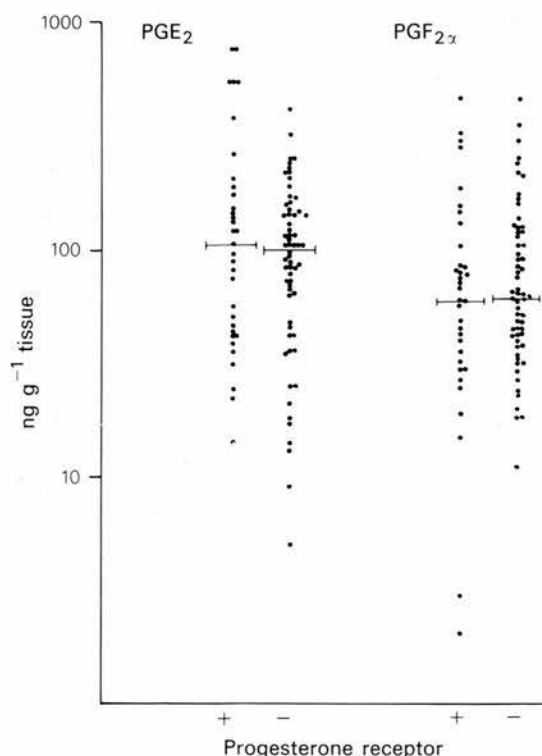


Figure 5 Levels of prostaglandins in progesterone receptor positive (+) and negative (-) tumours. Lines represent median values. No significant difference between the groups by Wilcoxon Rank Test.

It is almost impossible to measure "in situ" levels of prostaglandins within tissue preparations because prostaglandins are not stored in cells but are synthesised rapidly in response to stimuli. Biopsy, processing and homogenization of tumour specimens represent stimuli which would result in production of large amounts of prostaglandins. Determination of prostaglandins in tumours can, therefore, only be directed towards assessing the potential for prostaglandin production rather than measurement of endogenous levels (Green, 1979). Two types of technique have been adopted in this respect. Tumour may be homogenized directly in ethanol to obtain "basal" levels or incubated with or without added precursor to determine "synthesised" levels. It is not clear which technique more accurately reflects tumour potential for producing a local environment of prostaglandins or indeed if either reflects "in situ" activity. "Basal" levels will include both the normal content of tumour cells and material synthesised between biopsy and inactivation of synthetic enzymes during homogenisation (Bennet, 1982). The level of

prostaglandin "synthesized" will depend critically on precautions taken to protect the highly labile prostaglandin synthetase system and addition of arachidonate substrate may not mimic tissue levels of precursor. We have, therefore, chosen to measure basal levels of prostaglandins in the present study because this represents the least complicated and most practical method of studying prostaglandins in a large number of cancers.

Using these techniques, measurable amounts of PGE_2 and $\text{PGF}_{2\alpha}$ were detected in all tumours. Prostaglandins were identified on the basis of their molecular ions (at m/z 666 PGE_2 , m/z 653 for $\text{PGF}_{2\alpha}$), retention times and characteristic peaks. Amounts of PGE_2 varied from 7 to 762 ng g^{-1} tissue and those of $\text{PGF}_{2\alpha}$ from 3 to 475 ng g^{-1} tissue. These amounts are comparable to concentrations demonstrated by other methods (Bennet, 1982) and were higher than those found in benign and normal breast. A strong positive correlation was detected between levels of the two prostaglandins in individual breast cancers as has been previously observed (Fulton *et al.*, 1982). Although the variation in the amounts of both prostaglandins was large, we have been unable to identify factors accounting for this variation. Levels of tumour prostaglandins appeared to be uninfluenced by menopausal status of the patient, site of tumour biopsy or whether cytosolic steroid receptors were present.

The finding of no significant difference in tumour prostaglandin levels between pre, peri and post-menopausal patients agrees with the results of Rolland *et al.* (1980) who measured "synthesized" levels of prostaglandins in microsomal preparations. Fulton *et al.* (1982) failed to show an association with $\text{PGF}_{2\alpha}$ but reported significantly raised PGE_2 levels in postmenopausal women. It has been suggested that oestrogen receptor positive tumours synthesize greater amounts of prostaglandins (Campbell *et al.*, 1982). Others have been unable to show such a correlation (Rolland *et al.*, 1980; Bennet, 1982). However, in the study which reported a significant correlation, it was necessary to make a correction for tumour cellularity before the association became apparent. In the present study, a significant correlation was apparent between tumour cellularity and PGE_2 but not $\text{PGF}_{2\alpha}$ (Figure 6). However, multiple regression analysis of PGE_2 and $\text{PGF}_{2\alpha}$ on both oestrogen receptor and cellularity showed that oestrogen receptor had no significant effect on prostaglandins for given levels of cellularity (for PGE_2 $t=0.36$, for $\text{PGF}_{2\alpha}$ $t=1.12$). Indeed, correcting the results for tumour cellularity may be misleading because this would be based on the assumption that tumour cells within the biopsy were solely responsible

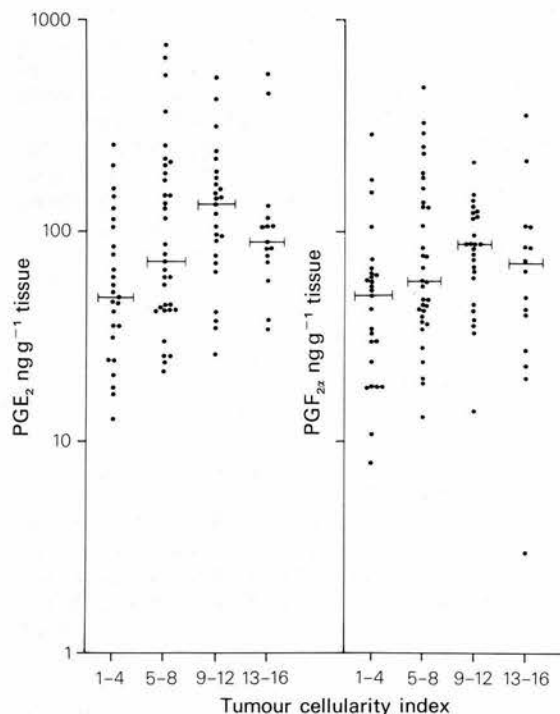


Figure 6 Levels of prostaglandins in tumours with different grades of cellularity. Cellularity was assessed as described by Hawkins *et al.* (1981) using a 4 point scale for both the proportion of tumour in the tissue specimen and for the proportion of malignant cells within the tumour itself. These are expressed as a product of the two scores to give a range of 1-16. (Score 1-4 therefore represents tumours with a low cellularity index, those with 13-16 the highest) PGE₂ correlates significantly with cellularity ($t=0.14$, $P<0.05$, by Kendal Rank Test). PGF_{2α} did not correlate significantly with cellularity ($t=0.08$).

whereas other cell types such as macrophages, lymphocytes, plasma cells and fibroblasts may also be producing prostaglandins. We have, therefore, preferred to express our results in terms of

prostaglandins extracted per weight of tumour tissue.

Variation in prostaglandins extractable from breast cancers may be due to non-specific, non-tumour factors such as the time interval between the biopsy and extraction, and the degree of trauma produced in obtaining the tumour samples. Enzymes associated with prostaglandin synthesis are particularly labile (Egan *et al.*, 1978) and it is essential to minimise any delay in tumour processing. Substantial amounts of prostaglandins may be generated in response to trauma (Green, 1979) and variation in degree of tissue trauma might be expected to be associated with differences in prostaglandins levels. In practice, it is difficult to eliminate tissue trauma as even gentle handling may stimulate biosynthesis of prostaglandins. In this series of tumours, clinical and pathological considerations determined the degree of mechanical trauma to which the specimens were subjected. However, no significant differences were detected between prostaglandins extracted from samples obtained at biopsy and those from mastectomy specimens.

It has been suggested that tumour prostaglandins are associated with prognosis and pattern of metastatic spread of breast cancer. At the present time, data from this study cannot be assessed for these parameters. Most tumours were obtained from patients with early breast cancer who have, as yet, only short follow-up. The absence of positive correlations with other factors of known prognostic value such as steroid receptors and lymph node status could mean that prostaglandins are either of independent significance or unrelated to prognosis. Valid assessment of the data will only be possible when further follow-up of the patients is available.

We would like to thank Professor A.P.M. Forrest for allowing us to study material from patients under his care, Dr J.M.J. Dixon for assessing the cellularity of each tumour and Dr R.A. Elton, Department of Medical Statistics and Computing for statistical analysis of the results.

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Cyclic AMP binding proteins in human breast cancer

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Summary The characteristics of a method for measuring cyclic AMP binding proteins in cytosols of human breast cancer are described. Using the assay, binding proteins were demonstrable in all of 100 tumour cytosols. Levels of binding in individual tumours varied from 0.8 to 15 pmol mg⁻¹ cytosol protein (mean value 5 pmol mg⁻¹ cytosol protein) and the dissociation constant ranged from 0.5 to 5.2 × 10⁻⁸ M (mean 1.73 × 10⁻⁸ M). Whilst replicate measurements within a single portion of tumour were reproducible (intra-assay coefficient of variation was between 4.5 and 7.8% and that for inter-assay variation was between 2.1 and 4.0%) there were often considerable differences in levels of binding proteins between different portions of the same tumour. Similar intra-tumour variations have been reported for other binding proteins and steroid receptors. The inter-relationships with such parameters may elucidate whether the differences are associated with variations in cellularity, cell type, or other specific factors.

In experimental animals, cyclic AMP binding proteins are implicated in the growth of mammary tumours (Cho-Chung, *et al.*, 1978b; Bodwin, *et al.*, 1980; Bodwin *et al.*, 1981). The corresponding evidence in human breast cancers has yet to be fully documented. In the present paper we describe a method for measuring total cyclic AMP binding proteins in human breast tumour cytosols and some characteristics of the assay.

Materials and methods

Reagents

(5'8'-3H) Adenosine 3',5'-cyclic phosphate, ammonium salt (45 Ci mmol) was obtained from Radiochemical Centre, Amersham, and radioinert adenosine 3',5'-cyclic phosphate, sodium salt from Sigma (Poole, UK). The following buffers were employed using analytical reagents - Buffer A 20 mM Tris, 0.25 M sucrose, 2 mM magnesium chloride, 1 mM calcium chloride, 10 mM potassium chloride, 16.26 mM HCl pH 7.5; Buffer B 55 mM potassium phosphate to which 11 mM theophylline was added immediately before use; Buffer C as Buffer B but with the addition of 10 mM magnesium chloride.

Tissues

Breast cancers were obtained at mastectomy or biopsy from patients with histologically proven disease. All material was transported on ice to a cold room and processed immediately unless stated

otherwise. The tumours represented 100 consecutive cases in which sufficient material was available for assay after tissue had been taken for routine histopathological examination and for oestrogen receptor analysis. Specimens were obtained from patients with T stage 1 to 4, although the number of T1 tumours was small.

Cytosol preparation

All procedures were performed at 0-4°C. Tumour was dissected from surrounding fat and connective tissue, finely cut with scissors and homogenized in Buffer A (w/v 1:10) using a Silverson homogenizer at maximum speed for 20 sec then 15 sec, with 1 min interval for cooling. The homogenate was centrifuged at 105,000 g for 1 h in a MSE Superspeed 50 centrifuge and the resulting supernatant was used as cytosol.

Binding measurements

Cytosol (50 µl) was incubated with 100 µl 5',8'-[3H] cyclic AMP (25 nM to give a final concentration in the incubation system of 10 nM) and Buffer B (100 µl) containing radioinert cyclic AMP (final concentration 0, 10, 20, 40, 80, and 10,000 nM). Each system was set up in duplicate and incubated at room temperature for 3 h. To separate protein-bound cyclic AMP from free nucleotide, 2 ml Buffer B was added to each tube. The contents were then mixed and filtered through a Millipore filter (HAWP 0.45 µm) at 5 mm Hg negative pressure followed by 20 ml Buffer C at 10 mm Hg negative pressure. The filters were transferred to scintillation vials and dried under a stream of air. Micellar fluor NE 260, Nuclear Enterprises (5 ml) was added to each vial. The vials were then

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incubated at 37°C for 2 h and radioactivity was measured in a Tricarb liquid scintillation counter (Packard).

Cytosol protein

The protein content of each cytosol was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Results

Assay conditions

Tumour cytosols were incubated with [³H] cyclic AMP in the absence and presence of 10,000 nM radioinert cyclic AMP for varying times, either at 0°C or 20°C. A typical result is presented in Figure 1. Maximum binding at 20°C was achieved by 2 h. Binding at 0°C was lower than at 20°C at each time point studied but was still increasing at 5 h incubation. Overnight incubation at either 0°C or 20°C produced similar binding (data not shown). For routine assays it was decided to incubate at 20°C for 3 h. The amount of binding under these conditions was linear with respect to increasing cytosol protein concentrations up to at least 3.0 mg ml⁻¹ (Figure 2). The effect of radioinert cyclic AMP on the binding of [³H] cyclic AMP is shown in Figure 3(a). Low concentrations of radioinert cyclic AMP were able to compete with [³H] cyclic AMP for binding, and there remained only a low level of non-specific binding in the presence of a thousand-fold excess of competitor. The data plotted according to Scatchard (1949), showed that the dissociation constant of binding was 2.7×10^{-8} M and that the maximum concentration of binding sites within the assay system was about 2.0 nM (Figure 3b). Similar results were also obtained by performing the assay with increasing concentrations of radio-labelled ligand and assessing the non-specific binding by including a 100-fold excess of cold competitor at each of these concentrations (data not shown).

Values in breast cancer cytosols

Cytosols from 100 primary breast cancers have been assayed for cyclic AMP binding proteins. The results are presented in Table 1, and the concentration of binding sites in individual tumours are plotted in Figure 4. All tumours showed cyclic AMP binding but levels varied greatly between individual tumours, from 0.8 to 15 pmol mg⁻¹ cytosol protein.

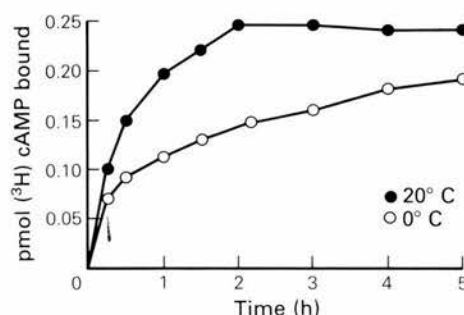


Figure 1 The effect of time of incubation on the binding of [³H] cyclic AMP to a cytosol of breast carcinoma either at 20°C (●) or 0°C (○). Each point represents the amount of [³H] cyclic AMP bound in the absence of radioinert cyclic AMP corrected for that in the presence of 10,000 nM cold competitor. Remaining assay conditions as described in **Materials and methods**.

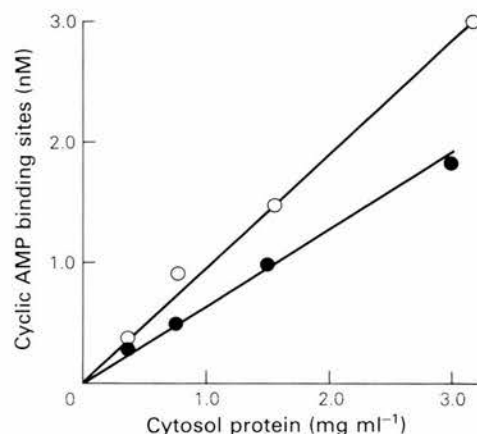


Figure 2 The effect of cytosol concentration on the binding of [³H] cyclic AMP to cytosols of 2 different breast carcinomas. Cytosols were prepared as described in **Materials and methods** and serially diluted to give the protein concentrations indicated. The diluted cytosols were incubated for 3 h at 20°C with increasing concentrations of radioinert cyclic AMP. The data were analysed by Scatchard plot and each point represents the maximum number of binding sites for each system.

Table 1 Levels and dissociation constants of cyclic AMP binding proteins in cytosols of 100 primary breast cancers

	Level pmol mg ⁻¹ cytosol protein	Dissociation Constant (M × 10 ⁻⁸)
Mean ± sd	4.99 ± 3.07	1.72 ± 0.96
Range	0.80–15.05	0.5–5.2

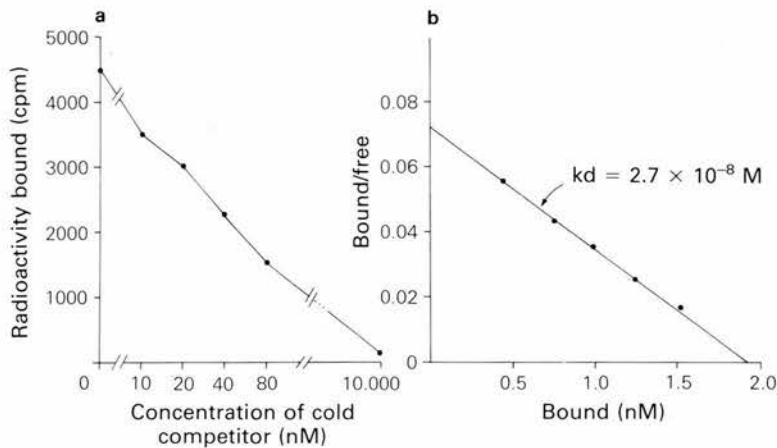


Figure 3 The effect of radioinert cyclic AMP on the binding of [^3H] cyclic AMP to a cytosol of human breast cancer. Assay conditions were as described in **Materials and methods**, data plotted as (a) radioactivity bound (b) according to Scatchard (1949).

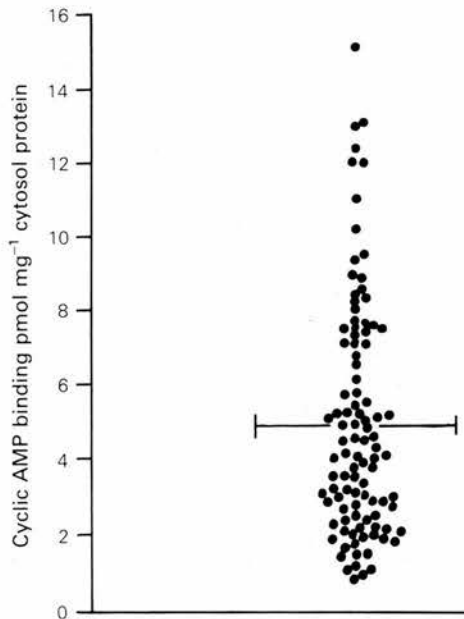


Figure 4 Levels of cyclic AMP binding proteins in cytosols of 100 primary breast cancers. Horizontal line represents mean value.

Reproducibility of measurements and effect of storage

In order to determine the intra-assay precision of cyclic AMP binding measurements in tumours, large breast cancers were finely minced. Five portions, each of $\sim 500 \text{ mg}$, were accurately weighed and cytosols were prepared separately and

assayed. Two tumours were processed in this way; one possessed a mean value for cyclic AMP binding proteins from the 5 replicate estimations of $1.38 \text{ pmol mg}^{-1}$ cytosol protein with an intra-assay coefficient of variation of 7.9%, the other cancer had a mean value of $7.48 \text{ pmol mg}^{-1}$ cytosol protein with an intra-assay coefficient of variation of 4.5%.

To ascertain the interassay variation, 3 tumours were divided into 5 portions, as described for the study of intra-assay variation. One portion of each tumour was assayed for cyclic AMP binding proteins immediately (day 0) and the remaining portions were stored in separate vials in liquid nitrogen for 1, 3, 7 and 14 days until assayed. The results are shown in Figure 5. There appeared to be no observable decline in level of binding proteins with storage, and considering measurements within the same tumour as replicate estimates, the interassay coefficients of variation were 2.1%, 2.5% and 4.0% (that these values are lower than those for the intra-assay variation is probably a reflection of the larger number of simultaneous estimations performed in the study of intra-assay variation).

An estimate of the variation in cyclic AMP binding protein levels within individual cancers was obtained by dissecting out portions of tumours from central, intermediate and peripheral zones across each of 12 large breast cancers. These were assayed by the routine method and the results are presented in Figure 6 as ratios of the values relative to that in the peripheral zone. Whilst the mean of the 12 values found in each tumour zone were similar (and hence the mean value for the zone ratio was unity), there were often large variations in cyclic AMP binding protein levels between different

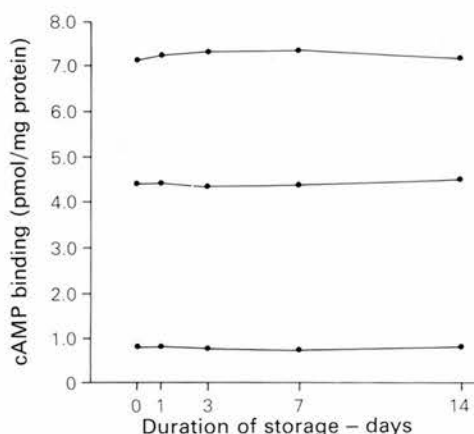


Figure 5 The effect of storage in liquid nitrogen on reproducibility of cyclic AMP binding protein levels in human breast cancers. Three separate tumours were studied.

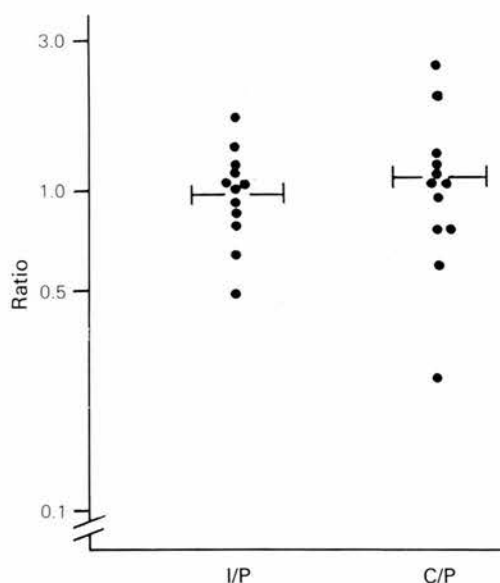


Figure 6 Variation in cyclic AMP binding protein levels across 12 breast cancers. For experimental details, see text. Results have been expressed as a ratio of the value obtained from the intermediate area (I) or the central area (C) to that in the peripheral area (P).

areas in an individual tumour. This variation was invariably greater when comparing central and peripheral zones.

Discussion

Measurements of cyclic AMP binding proteins in experimental animal cancers have yielded useful

information regarding the state of autonomy of the tumours (Cho-Chung *et al.*, 1980a; Bodwin *et al.*, 1980). Similar data in human breast cancers has not yet been fully assessed. In the present paper we describe the characteristics of an assay which might be used routinely to measure total binding sites for cyclic AMP in cytosols of human breast cancers.

This method involves incubating tumour cytosol with radioactively labelled cyclic AMP in the absence and presence of increasing concentrations of radioinert competitor. At 20°C, maximum binding was achieved by 2 h and was linear between cytosol protein concentrations of 0.4 and 3 mg ml⁻¹. Under these conditions the non-specific binding assessed by adding 1000-fold excess of radioinert cyclic AMP was negligible (<0.1% of the added radioactivity). The binding capacity was not affected by storage up to 14 days in liquid nitrogen. The intra-assay coefficient of variation, as determined on aliquots from minced large tumours, was between 4.5 and 7.9%, and the inter-assay value was between 2.1 and 4.0%. These results are similar to those obtained by others using a different method (Kvinnslund *et al.*, 1983).

Using the present method, cytosols of 100 human breast cancers have been assayed for cyclic AMP binding. All possessed binding activity, levels varying from 0.8 to 15.0 pmol mg⁻¹ cytosol protein (mean value 5 pmol mg⁻¹ cytosol protein). These values fall within the range for human breast cancer cytosols reported by others using different methods (Eppenberger *et al.*, 1980; Kvinnslund *et al.*, 1983), and are also similar to those found in rat mammary tumours (Cho-Chung 1978a; Cho-Chung *et al.*, 1978b). The mean dissociation constant of 1.73×10^{-8} M is also in keeping with data from experimental animal tumours (Cho-Chung *et al.*, 1978b).

It remains to determine which factors influence the levels of cyclic AMP binding proteins in cytosols of individual human breast cancers and, in particular, whether these levels are related to prognosis or endocrine responsiveness, as has been suggested by others (Kvinnslund *et al.*, 1983) and as is the case in rat mammary tumours (Cho-Chung 1978a, b, 1980). Assessments in breast cancers will have to take into account the variation in cyclic AMP binding proteins between different areas of the same tumour. Data from the present study shows that there may be considerable differences in the level of cyclic AMP binding between each of three different areas (central, peripheral and intermediate) of large tumours. No consistent pattern of variation across the tumours was evident and the mean value for cyclic AMP binding in this group of cancers was similar, irrespective of the area of tumour upon which the estimation was performed. At present, it is not known whether these

differences within tumours are associated with variations in cellularity, cell type or other factors. Similar intra-tumour variations have been noted with other binding proteins such as the oestrogen receptor (Hawkins *et al.*, 1977; Silversward *et al.*, 1980) and the inter-relationship with these different types of binding protein may help to elucidate the problem.

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Short Communication

Carcinoembryonic antigen (CEA) in explants of human breast cancer: comparison of immunohistochemical detection and release during short-term culture

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A large proportion of human breast cancers produce CEA (Heyderman & Neville 1977, Wahren *et al.*, 1978, Cove *et al.*, 1979). Although levels in plasma of patients with breast cancer have only a limited use in diagnosis and monitoring progression of the disease, (Chu & Nemoto 1973, Coombes *et al.*, 1980; National Institutes of Health Consensus Development Conference Statement 1981) measurement of CEA at the tumour level may provide a useful marker of tumour activity *in vitro*. We therefore measured CEA in media from cultured explants of human breast cancers and detected the marker in 75% of tumours (Miller *et al.*, 1980). However, these measurements give no indication of the proportion and type of cells producing CEA within the explants. This information may be obtained by histochemical staining techniques and the purpose of this study was to compare the quantitative release of CEA from explants into media during culture with the results from immunoperoxidase staining for CEA.

Histologically-proven breast cancers were obtained from 54 patients at mastectomy. Of these, 4 were classified as infiltrating lobular carcinomas and 50 infiltrating ductal carcinomas (2 mucinous, 1 medullary and 47 showing no special features). Fifty specimens were from the primary tumour and 4 were from invaded lymph node.

Each tumour was cut into explants measuring $4 \times 1 \times 1$ mm. Four weighed explants were placed on lens paper mounted on stainless-steel grids in each of 3 petri-dishes. Waymouths 17B 725/1 medium (2 ml) containing L-glutamine (2 mM), 20 mM Hepes and insulin ($10 \mu\text{g ml}^{-1}$) was added and the dishes incubated in an atmosphere of 95% O_2 /5% CO_2 for 24 h at 37°C. Culture medium was removed and assayed for CEA by radioimmunoassay.

For radioimmunoassay, CEA was prepared from liver secondaries of primary colonic cancer by perchloric acid extraction (Krupey *et al.*, 1968), followed by chromatography on columns of DEAE-cellulose, CM-cellulose, concanavallin A-Sepharose and Sepharose 6B. Rabbit antiserum to the purified CEA was absorbed extensively against perchloric acid extracts of normal human liver, lung, spleen and serum. Using purified CEA both as standard and for labelling (Sturgeon, 1978), a direct double-antibody RIA for CEA based on that of Egan *et al.* (1972) was developed. The assay was standardized using the British Standard for CEA (Laurence *et al.*, 1975), 1 ng of working standard being equivalent to $0.0058 + 0.0004$ units of the British Standard. Standard curves were prepared in culture medium. The working range of the assays for undiluted samples was from $3-70 \mu\text{g l}^{-1}$ and intra-assay precision averaged over this concentration range was 11.4%. Inter-assay precision was 10.0%, 8.3% and 6.4% at concentrations of 10, 25 and $50 \mu\text{g l}^{-1}$, respectively.

In order to assess immunohistochemical staining for CEA, 4 explants ($4 \times 1 \times 1$ mm) were cut from each tumour from the area immediately adjacent to that used for tissue culture. The method used was as described previously (Walker 1980). Tissue was fixed in 4% formaldehyde in 0.15 M sodium chloride, routinely processed and embedded in paraffin wax. Sections were treated with 0.1% solution of trypsin (Difco 1:250) for 10 min. Rabbit anti-CEA serum (Dako-immunoglobulins A115), which had been absorbed against non-specific cross-reacting antigen, was applied followed by the 3-stage peroxidase anti-peroxidase complex method. Controls used were normal rabbit serum in place of the primary antiserum; anti-CEA serum absorbed with CEA; and positive (carcinoma of colon) and negative (normal breast) control tissues.

Staining was assessed as negative (–ve), positive (+ve) or \pm if only very occasional cells had reacted (<5%).

Of 54 tumours, media from 41 contained measurable amounts of CEA in all replicate cultures

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after incubation; CEA was not detected in any culture of the remaining 13 tumours. In tumours producing CEA, the mean level varied from 3.0–1200 ng ml⁻¹ culture fluid. Immunohistochemical staining for CEA gave positive results in 34 cancers. There was no staining in the appropriate controls. In all carcinomas which gave a reaction there were variable numbers of +ve and -ve cells. An example of tumour graded +ve is shown in Figure 1. The site of staining within cells was either predominantly at the periphery with a faint granular cytoplasmic reaction, or throughout the cytoplasm with occasional focal intensities. All 14 tumours which were graded +ve produced CEA in culture as did 17 of the 20 graded \pm ve (Table). However, one half of the tumours which were -ve by the immunoperoxidase method had consistently detectable, but low levels of CEA in media after culture (Figure 2). Concentrations of CEA produced during culture were significantly higher ($P < 0.05$) in tumours graded histochemically +ve as compared

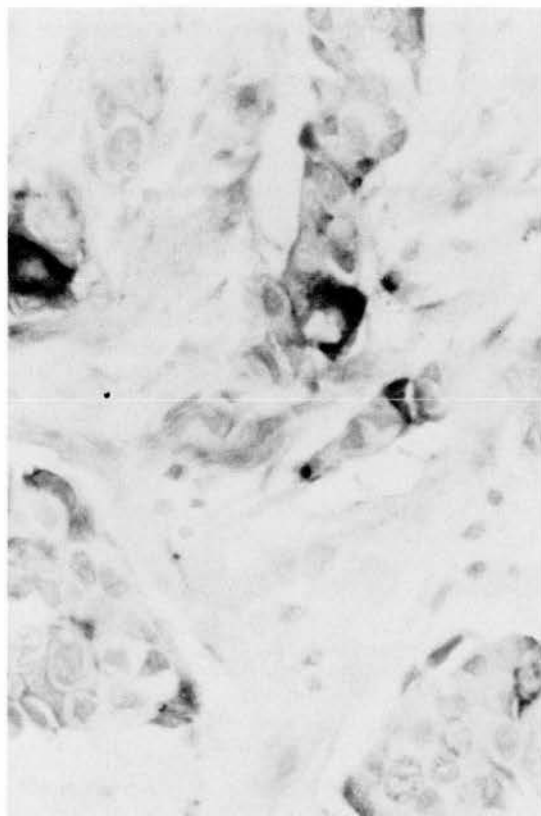


Figure 1 Small groups of breast carcinoma cells, with several individual cells having a prominent positive reaction for CEA as diffuse or focal staining within the cytoplasm. Immunoperoxidase, $\times 480$.

Table 1 Correction between immunoperoxidase and detection of CEA in media from cultured explants

		Immunoperoxidase grading		
		-ve	\pm ve	+ve
Media from cultured explants	-ve	10	3	0
	+ve	10	17	14

$\chi^2 = 8.75$, $P < 0.003$ trend

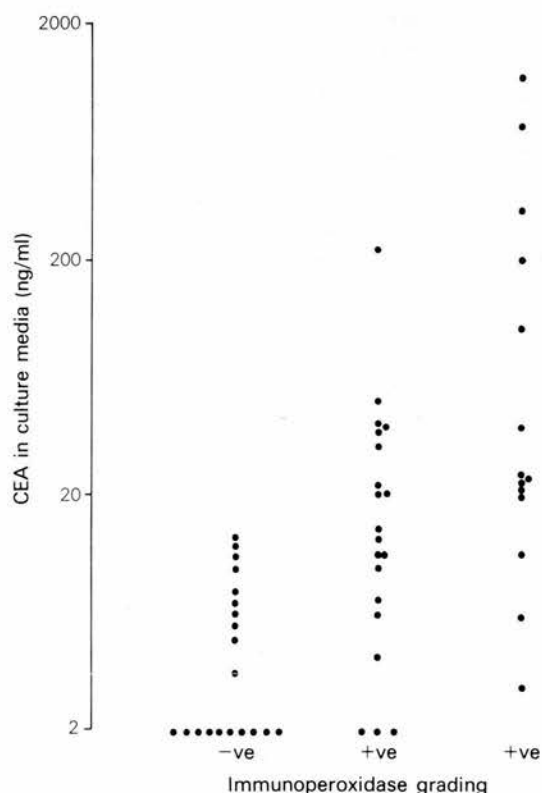


Figure 2 CEA levels in culture media from tumours subdivided according to immunoperoxidase grading. Significant differences by Wilcoxon rank testing. +ve against \pm ve $P < 0.05$, \pm ve against -ve $P < 0.01$.

with those in tumours graded as \pm ve, which in turn were significantly higher than those graded -ve ($P < 0.01$).

Approximately 75% of human breast cancers maintained in short term organ culture released measurable amounts of CEA into the media. This incidence is in agreement with that reported for tumour extracts (Cove *et al.*, 1979). From these data, however, it is not possible to indicate which cells or cell types are responsible for the production

of CEA. Such information can be obtained from histochemical studies and this study shows that explants from 34/54 tumours investigated possessed cells which stained positively for CEA using an immunoperoxidase technique. The incidence of detection of CEA by the immunoperoxidase technique ranged in previous studies from 1.5% (Goldenberg *et al.*, 1978) to 83% (Heyderman & Neville, 1977). These variations are probably due to the method employed and to the nature of the antiserum. Primus *et al.*, (1980) now advocate the use of the peroxidase-antiperoxidase complex method in preference to the bridge technique which they had used previously. Like studies on other human cancers, the present results showed that only a proportion of the cells within the tumour explants stained positively for CEA. The presence of CEA staining within the cytoplasm supports its role as a secretory product.

Only 3/34 tumours with immunoperoxidase staining failed to release CEA into the culture media. These 3 tumours contained very few positively staining cells and as different explants were used for culture, it is possible that this discrepancy reflects heterogeneity within the tumour.

In view of the significant positive correlation between the immunoperoxidase technique and the presence of radioimmunoassayable CEA in culture media, it is likely that the cells stained immunocytochemically are responsible for the production of CEA during culture. Measurement of CEA in the culture media may therefore offer a means of monitoring the activity of these cells *in vitro*.

Explants from half of the immunohistochemically-negative tumours released CEA into the medium during culture. The amounts released were relatively small and, in spite of using the

peroxidase-antiperoxidase method, it is probable that the technique is insufficiently sensitive in formalin-fixed paraffin embedded tissue to detect production of small amounts of CEA. These immunohistochemically false-negative tumours represented 10/15 carcinomas which, while producing CEA in culture, did so in amounts $< 2 \mu\text{g g}^{-1}$ tumour. This level of sensitivity for the immunoperoxidase method would be in agreement with that quoted by Goldenberg *et al.* (1978) for similar material from other tumours. The variation in results may also represent differences in the nature of the antiserum used for the immunohistochemistry and the RIA, and/or heterogeneity within the tumour as previously mentioned. In order to assess heterogeneity across the tumours, sections from the paraffin blocks used originally for histological grading were stained immunohistochemically. Although 6/45 tumours examined changed grading on the basis of this larger section, only one carcinoma which released CEA during culture but graded -ve by histochemical staining of the explant, was classified \pm ve in the tumour slice.

It is concluded that RIA of media from cultured tumour explants provides a sensitive quantitative estimate of CEA production by breast carcinomas, and that immunohistochemical staining for CEA indicates the proportion and nature of the cells whose activity is being measured within the tumour. It is suggested that, in order to monitor tumour *in vitro* activity by CEA measurements, both methods should be used in combination.

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MILK PROTEINS AND BREAST CANCER

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INTRODUCTION

The principal function of the breast is the synthesis of proteins, fats and carbohydrates, all components of milk. The proteins synthesized by the breast, during lactation, are the final products of highly differentiated glandular tissue. Therefore, they differ from the first tumour markers used such as CEA, AFP, HCG and its subunits, which are proteins secreted by young, most often embryonic or cancerous, cells. In contrast, milk proteins are synthesized by adult cells arrested at a complete stage of development. Mammary tissue from primates (Kleinberg and Todd, 1978) and humans (Bussolati *et al.*, 1975) has been shown to produce and contain lactalbumin and casein even in virgin, nulliparous and multiparous breasts. Thus, the breast cells are able to produce milk proteins at various times of life, other than during gestation and lactation.

Several examples exist of the use of tissue secretory products as an index of tumour activity (Laurence and Neville, 1977). The development of such systems for milk proteins produced by breast cancer, needs the maintenance of synthesis material in cancerous cells. Lactalbumin (Kleinberg, 1975; Kleinberg and Todd, 1978; Woods *et al.*, 1979), and casein (Bussolati *et al.*, 1975; Herbert *et al.*, 1978) have been observed in cancerous breast cells. Nevertheless, the frequency of lactalbumin production by cancer cells is lower than by normal cells and the

induction of lactalbumin production by prolactin can be stimulated in normal but not in malignant breast tissue (Wilson *et al.*, 1980). The milk proteins are produced less by cancer cells. But these milk proteins could be an index, during the woman's life, of breast activity and secretion. They are an index of, on the one hand, a particularly favourable hormonal climate for the breast cells to synthesize and secrete material in the mammary ducts, and, on the other hand, of their mitotic activity. Petrakis *et al.* (1975) showed a correlation between breast secretory activity and breast cancer risk. Furthermore, the breast secretion from nipple aspirates could have mutagenic activity (Petrakis *et al.*, 1980).

Thus, the use of milk proteins in breast oncology may be of interest in two potential areas: that in the cancer cells synthesize a protein which is biological parameter of tumoral activity, or that this protein is an indication of a breast in an hormonal condition of cancer transformation risk.

Two milk proteins are studied. Firstly, lactalbumin, with a molecular weight of 15,000 daltons (Brew *et al.*, 1970), has a biological role to provide a rapidly responding control mechanism acting on the lactose synthetase in the milk (Fitzgerald *et al.*, 1970). Lactalbumin is a major component of the milk (1-2 mg/ml), and the final product of a highly differentiated adult tissue.

The second protein studied, the sweat α_2 -globulin, was described by Jirka (1968). This mucoprotein is present in a number of external secretions: saliva, tears, cerumen, colostrum and milk. This product of the mammary and sweat glands which have a similar embryological origin, has been purified from breast cyst fluid of patients with breast cyst disease: GCDP 15 (Haagensen *et al.*, 1979), and GCDP 70 (Zangerle *et al.*, 1981). It is a minor component of milk (1-2 μ g/ml) and a major one of breast cyst fluid and breast secretion (1-10 mg/ml); its biological role is still unknown.

We have refined and assessed a radioimmunoassay for lactalbumin and GCDP 70. We used this assay to study these two milk proteins in serum from different populations, in culture fluid from breast explants, in breast cyst fluid from breast cystic disease patients, and in breast secretion from nipple aspirates.

MATERIAL AND METHODS

Radioimmunoassay

Lactalbumin Assay

Lactalbumin was purified, according to the method of Schultz and Ebner (1977) from milk of lactating women collected between post-partum days 4 and 10. The protein obtained had a molecular weight of 15,000 daltons and the enzymatic activity for lactose biosynthesis by transferring galactose, from UDP-galactose, on glucose. This lactalbumin was used as a tracer after labelling with 125 I (Greenwood *et al.*, 1963) and as a reference preparation. Other milk proteins, known tumour markers and egg lysozyme serum proteins from children do not cross-react with human lactalbumin. The sensitivity of the assay is 75 pg/ml and the interassay variation coefficient less than 4%. In the cases where human immunoglobulins capable of binding the labelled lactalbumin were present, we used the method of Woods *et al.* (1978) to neutralize them.

GCDFP 70

Gross cystic disease fluid protein was extracted from breast cyst fluid of women with breast cystic disease. In biological conditions the molecular weight is 70,000 daltons. This molecule was used as a tracer and as a reference preparation. Any other milk proteins, tumour markers such as CEA, HCG, β and α HCG or serum proteins from normal males do not cross-react in the immunological reaction. The sensitivity of the assay is 800 pg/ml, and the interassay variation coefficient is less than 5%.

Sera Studied

Normal Populations

Lactalbumin and GCDFP 70 were assayed in the serum of healthy subjects: 200 adult women, 200 adult men, 20 prepubertal boys and girls, five young women every 2 days during normal menstrual cycle, 55 gestating women and 19 lactating women during the 6 days post-partum.

Benign Disease Patients

Non-breast benign disease. There were 438 patients investigated with non-breast benign disease: 62 were bearing different digestive diseases (except viral hepatitis); 21, lung disease; 112, cardiovascular diseases; 23, infectious diseases; 36, endocrinological diseases; and 27, urogenital diseases.

Breast benign disease. There were 123 adult women investigated with breast benign disease: 74 were bearing non-cystic disease, such as fibroadenoma, and 49 had breast cystic disease.

Malignant Disease Patients

There were 105 patients investigated with breast cancer at different stages of evolution; 51 with lung cancer, 62 with digestive cancer, and 32 with different site cancers.

Breast Cyst Fluid

Forty-two breast cyst fluid samples were obtained by a needle aspiration of the cyst from women with breast cyst disease. Multiple cysts from the same patients were analysed in nine women.

Breast Secretion

Nipple aspirates (100–250 μ l) gave breast secretion in 100 women without clinical evidence of breast disease.

Breast Culture

Tumours are cut into explants. Four weighed explants were placed on a lens paper, mounted on stainless steel grids in Petri dishes and cultured for 24 h at 37°C in 2 ml Waymouths 17 β 725/l medium, containing normal glutamine, 20 mM Hepes and insulin (10 μ g/ml) with an atmosphere of 95% oxygen/5% CO₂. After culture, media are removed and deep frozen at -40°C until assayed

for tumour markers. The explants are weighed, pulverized in liquid N_2 , and extracted with a further 2 ml of culture medium. After centrifugation the resultant cytosol is removed and used for the estimation of milk proteins.

RESULTS

Sera from Normal Populations

Children

GCDFP None of the 20 male and 20 female children before puberty presented serum values of GCDFP higher than 5 ng/ml.

Lactalbumin. Twelve of the 20 boys (60%) and eight (40%) of the girls did not have a detectable value of lactalbumin. No value exceeded 3 ng/ml.

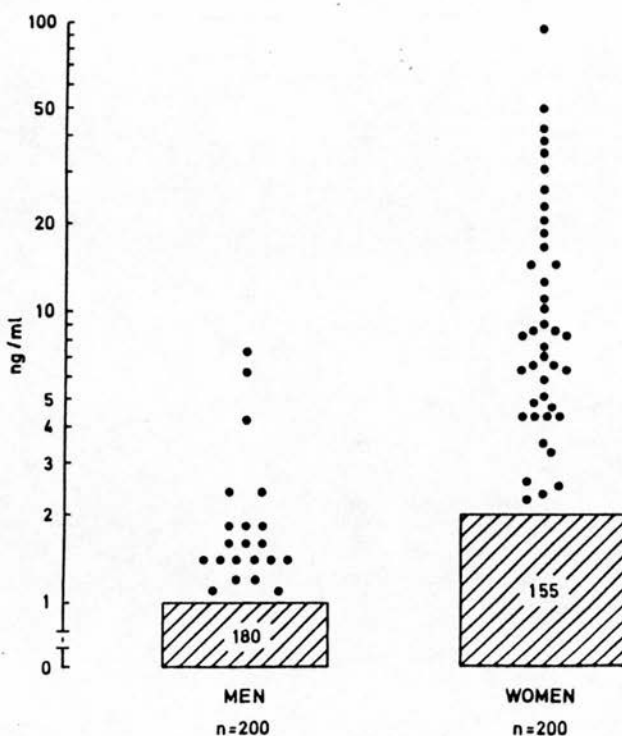


Fig. 1. Individual values of lactalbumin in healthy men and women.

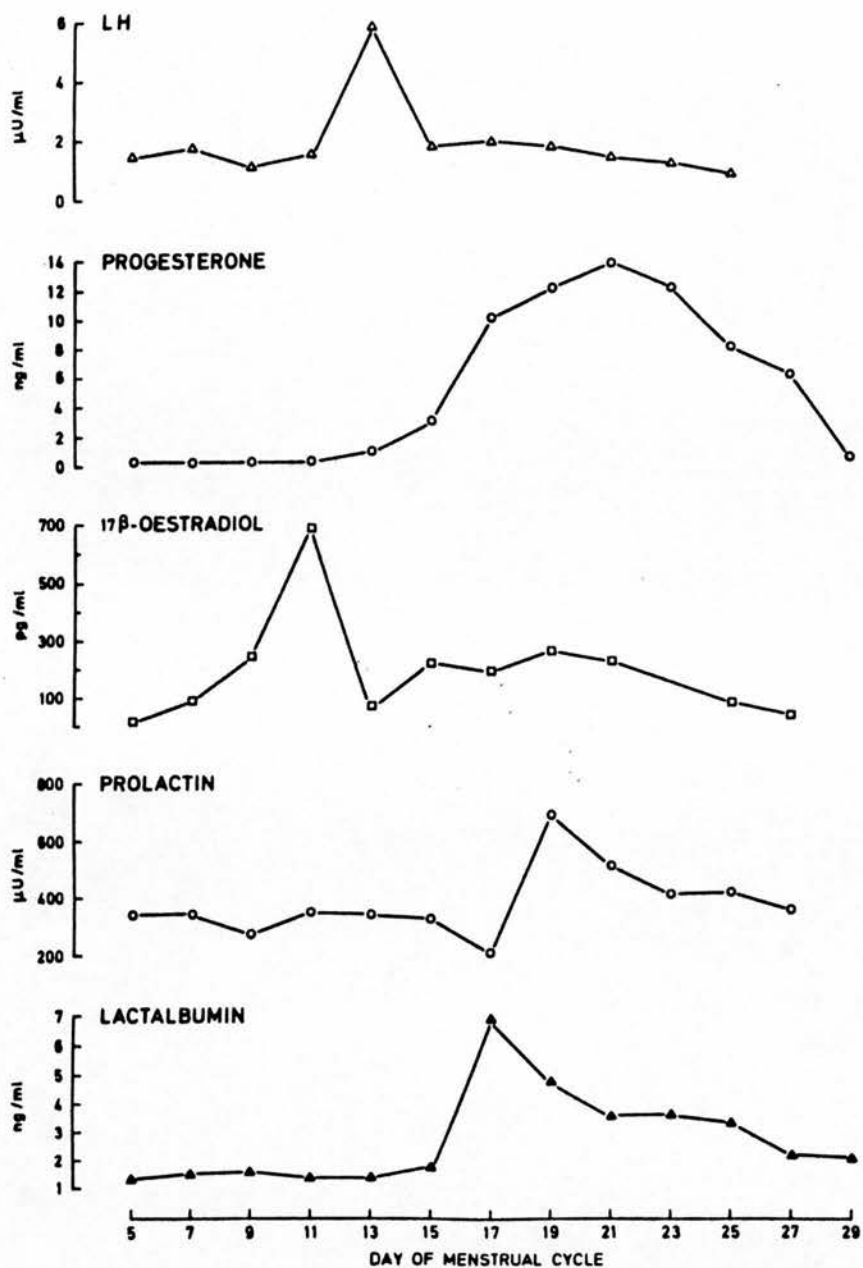


Fig. 2. Serum concentration of luteinizing hormone, progesterone, 17 β -oestradiol, prolactin and lactalbumin throughout a menstrual cycle. Samples were withdrawn every 2 days from a 20-year-old fasting woman.

Healthy Adults

GCDFP. Four of the 400 healthy males and females presented values of GCDFP higher than 5 ng/ml. There is no correlation between the detectable value and the sex repartition. No correlation has been found between blood groups.

Lactalbumin. Lactalbumin was detected in the serum of 92/200 men (47.5%) with levels equal to or less than 4 ng/ml. Three of the 200 (1.5%) had levels higher than 4 ng/ml. No value exceeded 7.2 ng/ml (Fig. 1).

Out of 200 women, 124 (62%) had detectable values of lactalbumin with a wider range than in men (Fig. 1). Those women with values higher than 4 ng/ml represented 19.5% of the group. In this last group of women, the mean value of prolactin ($694.32 \mu\text{IU/ml} \pm 342$) is higher than the mean value found in women without detectable values of lactalbumin ($483 \pm 268 \mu\text{IU/ml}$). There was no statistical difference concerning the value of 17β -oestradiol, progesterone between the group of women with detectable lactalbumin values and the group without detectable lactalbumin values.

Menstrual cycle. Of the five women studied throughout their menstrual cycle, three had detectable values of lactalbumin. Each presented a peak of LH between days 13 and 19 of the cycle (Fig. 2). In positive lactalbumin women, the lactalbumin values increased quickly at day 2 of the luteal phase, then decreased progressively. The mean value of lactalbumin was significantly higher during the luteal phase than during the follicular phase (Fig. 3).

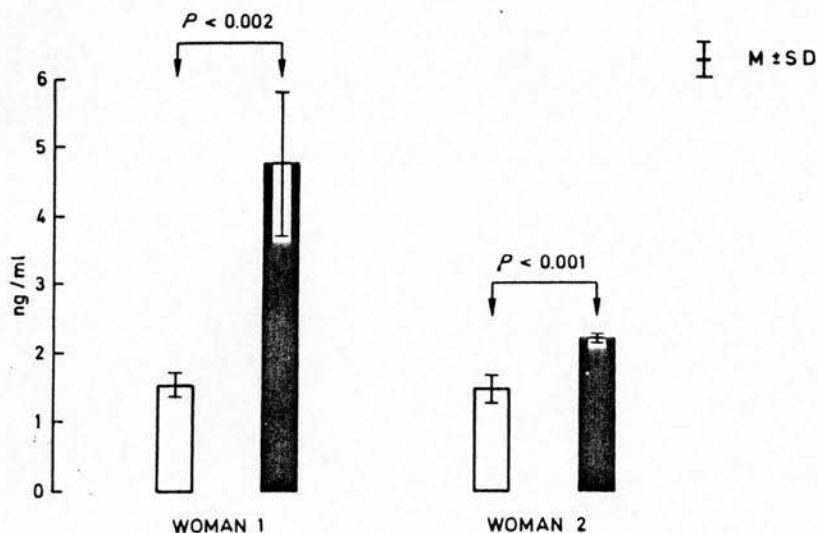


Fig. 3. Mean lactalbumin levels, with standard deviation, during the follicular (open columns) and the luteal phase (closed columns) of two women normally cycled.

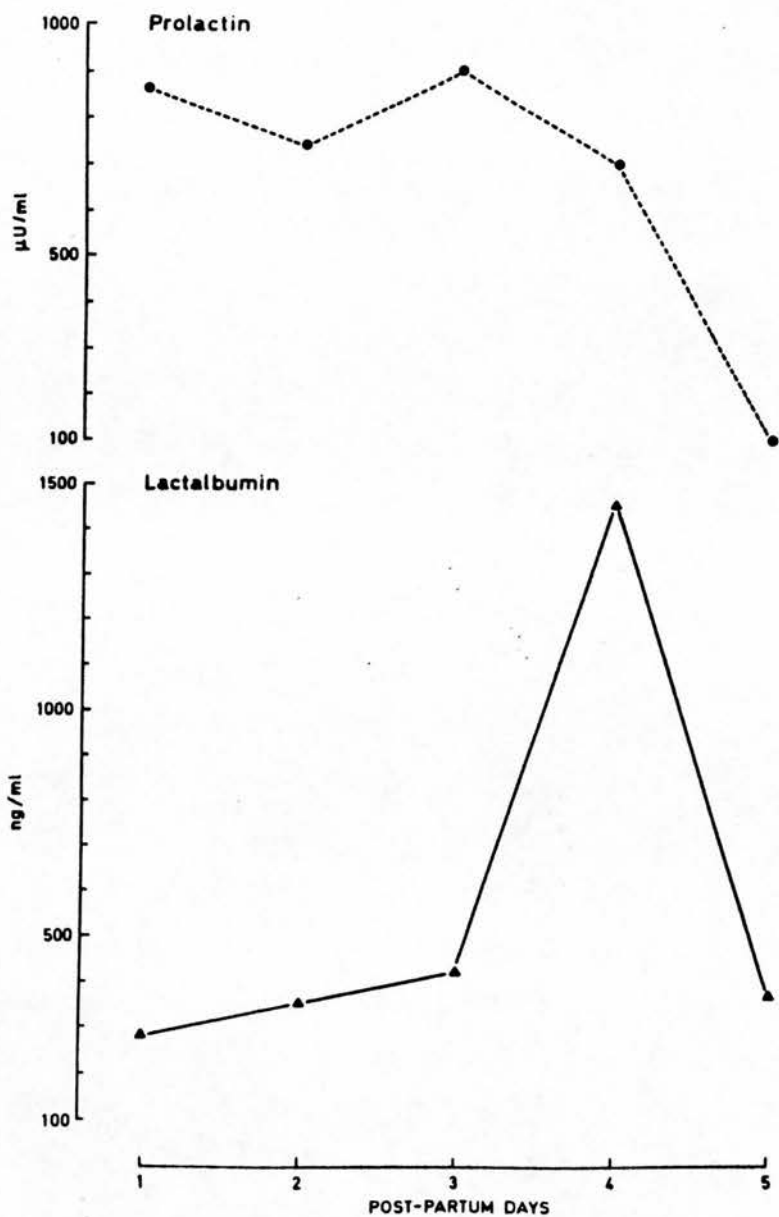


Fig. 4. Evolution of prolactin levels and lactalbumin levels in the post-partum period of a normal lactating woman.

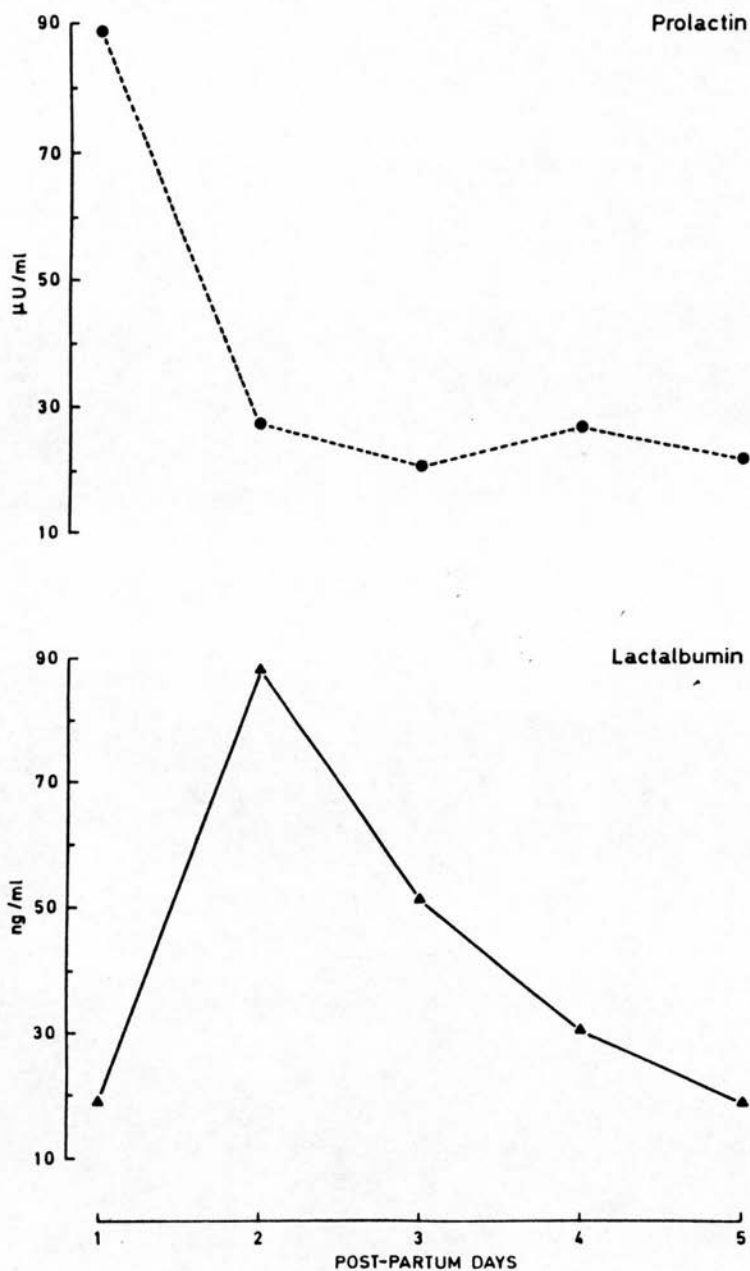


Fig. 5. Evolution of prolactin levels and lactalbumin levels in the post-partum period of a non-lactating woman with bromergocryptin (Sandoz).

Gestating women. No serum from gestating women had GCDFP values higher than 5 ng/ml. Lactalbumin was detected in all the sera of gestating women with a mean value of 120 ng/ml at the end of gestation.

Lactating women: normal lactation. In 17 women the prolactin and the lactalbumin levels were determined each morning, 15 min before the second suckling, during the first 6 days following delivery. Mean prolactin levels increased to reach their maximum on day 2 of the post-partum, then progressively decreased. The lactalbumin levels increasing during lactation, reached a maximum on day 4 after delivery and decreased thereafter (Fig. 4).

Non-lactating women. Two women who did not wish to breast feed, received bromergocryptine (Sandoz). Prolactin levels decreased to normal values after 3 days. Nevertheless, the lactalbumin levels remained low, although reaching a small peak on day 2 and then decreasing but with levels ten-times less than the levels found in lactating women (Fig. 5).

GCDFP was found only in one woman without fluctuation, according to the lactation time.

Patients with Benign Disease

Non-breast Benign Disease

In 315 patients with non-breast benign disease, the incidence of GCDFP higher than 5 ng/ml was 4/315 (1.2%) and the incidence of lactalbumin higher than 4 ng/ml was 5/315 (2.5%).

Benign Breast Disease

The incidence of pathological values of GCDFP (> 5 ng/ml) was clearly different in patients with breast cyst disease (27/49, 55%) from the incidence found in patients with non-breast benign disease (1/74, 1.3%). The incidence of high lactalbumin levels in breast cyst disease patients (11/49, 22%) was also quite different from that in non-cystic breast disease (9/74, 12%) (Fig. 6).

Patients with Cancer

Non-breast cancer. The incidence of pathological values of GCDFP and lactalbumin in lung, digestive and different site cancer was 10/145 (6.9%) and 2/145 (1.4%), respectively.

Patients with breast cancer. Whatever the state of the disease, the incidence of lactalbumin values higher than 4 ng/ml was low—5%. In 105 women with breast cancer at different stages of the disease, the incidence of pathological values of GCDFP was 64/105 (61%).

Breast cyst fluid. The levels of GCDFP in cyst fluid were high ranging from 2 to 10 mg/ml. Multiple cysts were analysed in nine women and the concentration of GCDFP was in the same range in cysts of the same individual. The range of lactalbumin values was lower than in milk—from 0.1 to 150 μ g/ml.

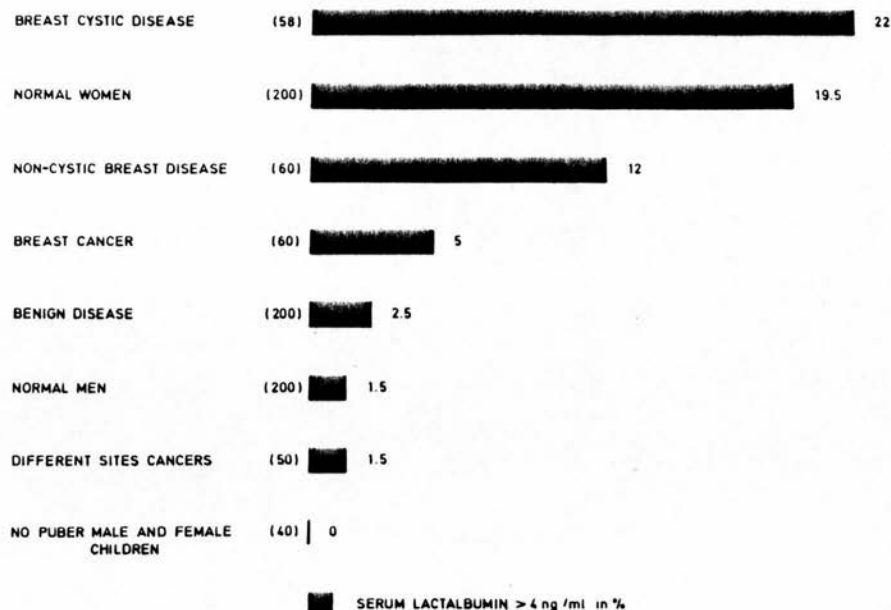


Fig. 6. Incidence of lactalbumin levels higher than 4 ng/ml (%) in the serum of healthy, with benign disease and with cancer populations.

Breast secretion. The breast secretions of nipple aspirates had the same range of GCDFP concentration as in breast cyst fluid. The values of lactalbumin were lower and never exceeded 125 ng/ml (Fig. 7).

Culture medium. Seventy-five out of 84 human breast cancer explants produced GCDFP in the culture fluid with an extremely wide range of levels, varying from 0.4 to 125 ng/ml. Of the remaining nine, five were equivocal and four had no evidence of GCDFP production (Fig. 8). Lactalbumin was present in eight only (9.5%). In cultures producing GCDFP the levels were always much lower in media on day 2 compared with those on day 1 and values in the explants after culture were also very low. The combined levels in the culture media exceeded those present in the explants before culture; this may be evidence of *de novo* synthesis during culture. The levels of GCDFP 70 in the culture fluid were not related to:

- (1) the menopausal status of the patient,
- (2) tumour oestrogen receptor (Fig. 9);
- (3) whether the cancer tissue was derived from primary tumour or invaded node (Fig. 10); or
- (4) from patients with early or late disease.

Three explants from normal breast tissue did not produce GCDFP or lactalbumin in the culture fluid.

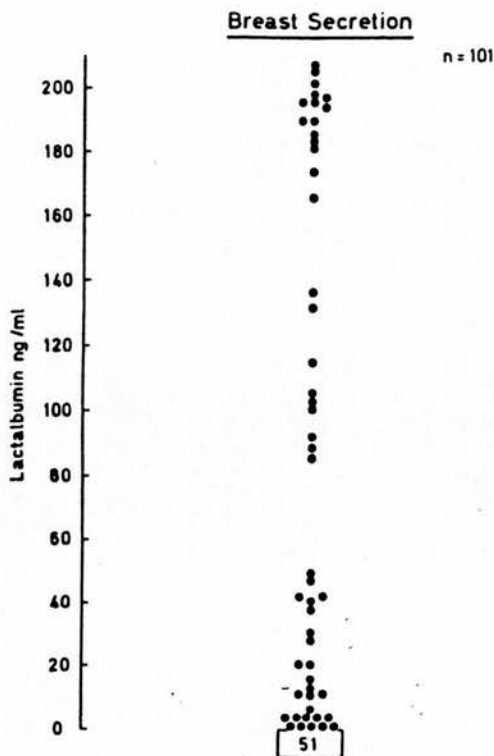


Fig. 7. Lactalbumin levels in breast secretion from nipple aspirates of 101 healthy women.

DISCUSSION

Lactalbumin

The purified molecule has the molecular weight and the biological enzymatic activity previously described (Schmidt and Ebner, 1971; Brew *et al.*, 1970; Fitzgerald *et al.*, 1970).

The levels found in the serum of normal population were similar to those assessed by Kleinberg (1975), Woods and Heath (1978) and Woods *et al.* (1978).

Lactalbumin can be considered as an index of the physiological activity of the breast.

(1) In lactating women, the serum lactalbumin level evolution is the same as the kappa casein evolution (Zangerle and Hendrick, 1976; Zangerle *et al.*, 1978; Franchimont *et al.*, 1979) with values comparable to those described by Martin *et al.* (1980). Like kappa casein, the lactalbumin does not follow exactly the evolution of prolactin.

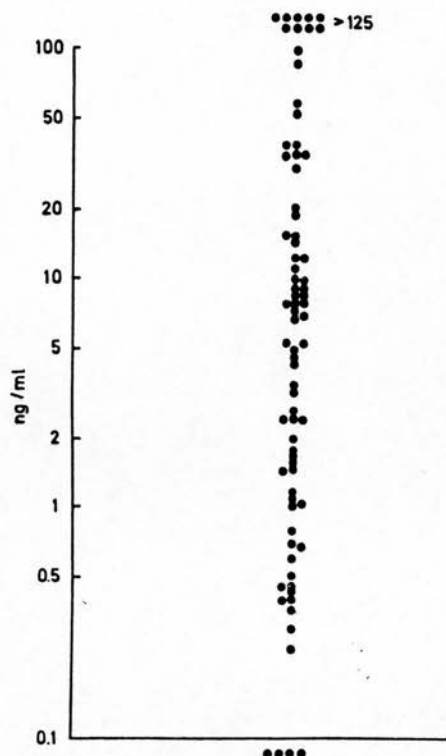


Fig. 8. GCDFP values in day 1 fluid of breast cancer explant cultures.

(2) During the menstrual cycle, when present (three out of five women), there is a cycle of lactalbumin with higher mean values during the luteal phase than those in the follicular phase, together with higher values of prolactin, 17β -oestradiol and progesterone.

(3) In a normal population, a sex difference, which does not exist among prepubertal girls and boys, appears between adult men and women (Fig. 1).

In women, the hormonal difference between the group with detectable lactalbumin and the group without detectable lactalbumin is only significant for prolactin.

Lactalbumin is not a marker of breast cancer since it is not found in breast cancer culture fluid and in breast cancer cell cytosols. The absence of lactalbumin in the culture could be attributed to the short time periods of culture and the complete absence of hormone in the fluid. Nevertheless, in the presence of cortisol (Ono and Oka, 1980), prolactin (Wilson *et al.*, 1980; Kleinberg and Todd, 1978) or oestrogen (Ip and Dao, 1978), cancerous cells do not product lactalbumin more than do normal cells.

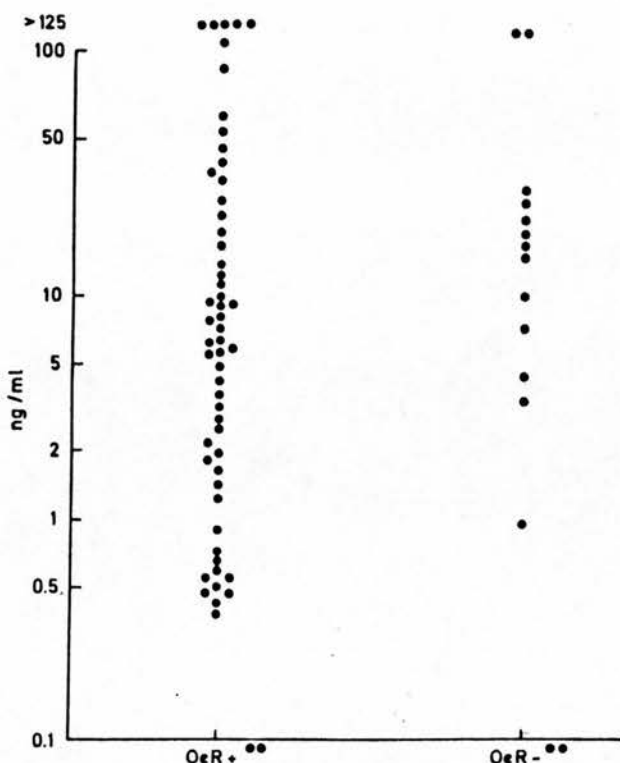


Fig. 9. GCDFP values repartition in day 1 fluid of breast cancer explant culture, following the presence (OeR⁺) or the absence (OeR⁻) of oestrogen receptor in the tumour.

In the serum of breast cancer patients, the incidence of lactalbumin values higher than 4 ng/ml is low—5%. The presence of lactalbumin in the serum could be related to the presence of high risk disease since 22% of patients with breast cyst disease have values higher than 4 ng/ml (Fig. 6). This cystic mastopathy has been associated with a tendency to hyperoestrogenism, frequently related to luteal insufficiency (Mahon *et al.*, 1973).

The presence of lactalbumin in normal breast secretion ($\rightarrow 125$ ng/ml) and in breast cyst fluid ($\rightarrow 125$ μ g/ml) shows the persistence of secretory activity of the breast during normal and pathological conditions.

Following these results, lactalbumin could be considered as an index of mammary function, but not as a marker for breast cancer. As its production depends on multiple hormones, its presence in the serum indicates a prevalence of hormones such as prolactin, acting on the breast metabolism, synthesis, secretion and mitotic activity or a difference in the sensitivity of the tissue leading to the same activation. In this condition, the presence of high values of lactalbumin in the serum could be considered as an index of risk. This hypothesis is re-enforced

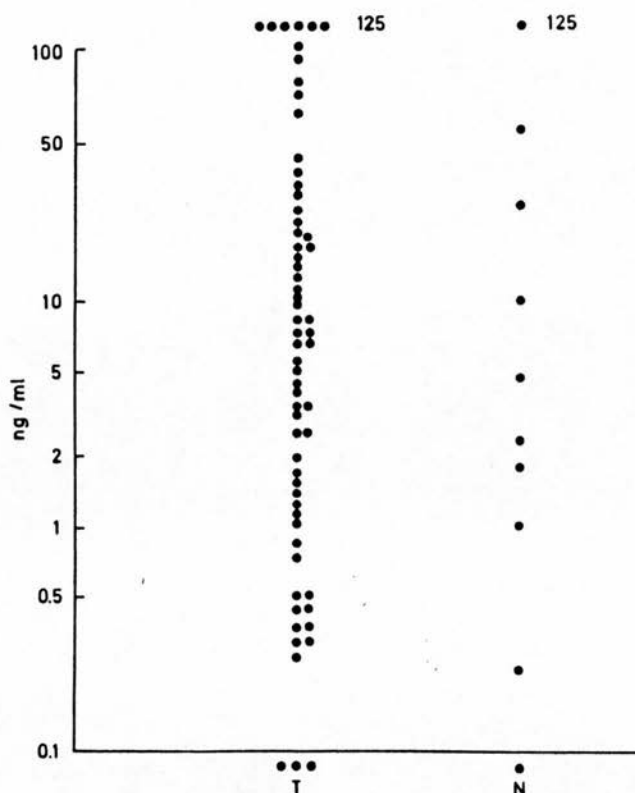


Fig. 10. GCDFP values repartition, in day 1 fluid of breast cancer explant culture from primary (T) or lymph node invaded (N).

by the studies of Patrakis (1973, 1980), which establish a relation between high secretion activity and breast cancer risk. Nevertheless, epidemiological studies are needed to confirm such an hypothesis.

GCDFP

GCDFP is quite different from lactalbumin: it has lower levels in milk and higher levels in breast secretion and in breast cyst fluid. GCDFP is not at all a marker of the function of the breast since it is absent in the serum of gestating or lactating women. Its level is not cyclated during the menstrual cycle.

GCDFP is produced essentially by cancer cells as shown by the 90% positive value in breast cancer culture medium and its presence in the cytosols of breast cancer cells. Normal mammary cells in culture do not seem to produce GCDFP.

In the blood, the presence of GCDFP can be correlated only with breast cyst disease (55%) and breast cancer (68%). It is not significantly found in any other benign disease or cancer from another origin. Therefore, GCDFP 70 can be considered as a marker of breast cancer. The reason why the mammary gland secretes this milk protein is still unknown.

It is interesting to note the association of pathological values for the two diseases: one benign with a high risk, the breast cystic disease, and the other, breast cancer.

The presence of blood values of GCDFP and lactalbumin in patients with GCDFP is an argument for the high metabolic activity of such a disease.

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LECTIN BINDING AND STEROID RECEPTORS IN HUMAN BREAST CARCINOMAS

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SUMMARY

A series of breast carcinomas of known steroid receptor status have been examined for evidence of binding of the lectins peanut agglutinin, soy bean agglutinin and wheat germ agglutinin. Correlations were found between oestrogen receptor status and reactivity of carcinomas to peanut agglutinin and soy bean agglutinin but these were not absolute. Wheat germ agglutinin binding was unrelated to the presence of oestrogen receptors. No relationship was evident between progesterone receptors and the binding of any lectin. It therefore seems unlikely that lectin histochemistry can replace steroid receptors as markers of hormone dependence in breast carcinomas.

KEY WORDS—Breast cancer, lectins, steroid receptors.

INTRODUCTION

The value of steroid receptors in breast carcinomas is now well established with regard to the prediction of patient response to endocrine therapy.¹ However the biochemical methods for assaying receptors are still not widely available. The detection in routinely fixed and processed carcinomas of proteins and/or glycoproteins whose synthesis is dependent upon hormones, and are therefore indicators of intact receptor mechanisms, could have obvious clinical applications. Since experiments had shown that binding sites for the lectin peanut agglutinin (*Arachis hypogaea*) in rat mammary tissue are hormone dependent, Klein *et al.*^{2,3} investigated the binding of this lectin to human breast carcinomas and concluded that it allowed a distinction to be made between hormone sensitive and non-sensitive tumours.

Peanut agglutinin (PNA) has a high affinity for the disaccharide β -D-galactosyl(1 \rightarrow 3)*N*-acetyl-D-

galactosamine.⁴ Binding of the lectin is a constant feature of normal human breast tissue, once sections have been treated with neuraminidase.⁵ The reactivity of carcinomas to PNA, in association with desialation, is related to differentiation.⁶ Another lectin whose binding to breast carcinomas is related to differentiation is wheat germ agglutinin (WGA),⁷ which has a high affinity for *N*-acetyl-D-glucosamine. Soy bean agglutinin (SBA) is a lectin which is specific for β -D-galactose and *N*-acetyl-D-galactosamine.⁸

In this study a series of carcinomas have been assessed for their reactivity to PNA, SBA and WGA, and the results have been compared with the oestrogen and progesterone receptor status of the tumours.

MATERIALS AND METHODS

Tissue was obtained from 50 primary infiltrating breast duct carcinomas, excised from the same number of patients at Edinburgh Royal Infirmary, and

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from three lymph node metastases from two further patients.

Samples taken for receptor analysis were placed on ice and processed immediately for homogenization at 4°C. Parallel slices were fixed in 4 per cent formaldehyde in saline and routinely processed.

Oestrogen receptors were assayed using a multiple point dextran coated charcoal method with Scatchard analysis.⁹ Progesterone receptor was also assayed by a dextran coated charcoal method with the use of R5020, a synthetic progesterone.¹⁰

Lectin binding was performed as previously described.⁶ The lectins were conjugated to horseradish peroxidase type VI (Sigma Chemical Co.). After section dewaxing and rehydration they were applied at the following concentrations: WGA 5 µg/ml; PNA and SBA 10 µg/ml. Sections to which SBA-peroxidase was to be applied were initially treated with 0.1 per cent trypsin, pH 7.8, for 30 min at 37°C. Parallel sections which were tested with PNA-peroxidase were treated with neuraminidase or acetate buffer at 37°C for 18 h prior to application of the lectin. Peroxidase was localized using the diaminobenzidine-hydrogen peroxide reaction. Controls were the incubation of adjacent sections with lectin absorbed with the appropriate sugar: PNA with 0.1 M D-galactose; SBA with 0.1 M D-galactose and 0.1 M N-acetyl-D-glucosamine; and WGA with 0.1 M N-acetyl-D-glucosamine. The lectin results were assessed without knowledge of the receptor status.

RESULTS

Carcinomas with oestrogen receptor < 5 fmol/mg protein were considered negative; between 5 and 19

fmol/mg protein 'receptor poor' and > 20 fmol/mg protein 'receptor rich'. The numbers in each category were 14, 9 and 30 respectively. Forty-one tumours were examined for progesterone receptor status; 29 were negative, i.e. < 5 fmol/mg protein and 12 were positive. One carcinoma was oestrogen receptor negative, progesterone receptor positive but the patient had received the anti-oestrogenic agent tamoxifen prior to biopsy. There were, therefore, 11 carcinomas which were oestrogen and progesterone receptor positive.

The pattern of lectin binding was assessed on the proportion of cells staining and was similar to that previously described.^{6,7} For PNA the reactivity after neuraminidase treatment was considered; the majority of cells stained in 27 carcinomas (categorized as many) mixtures of positive and negative cells were seen in 21 tumours (moderate) and in five carcinomas only a few cells reacted. Similar methods of assessment were made for SBA and WGA. Only four carcinomas had many cells reacting with SBA, so these were included with the 15 tumours having moderate numbers of positive cells; 16 carcinomas had a few reactive cells and 18 were negative. The results for WGA differed with 31 carcinomas having the majority of cells staining, 17 tumours showing moderate numbers of cells reacting and only five carcinomas having few reactive cells.

The comparisons of PNA, SBA and WGA binding with oestrogen and progesterone receptor status are shown in Table I. The results were analysed by χ^2 tests. Since the numbers in the oestrogen receptor 'poor' group were small two separate analyses were undertaken for all lectins in which this group was included with (i) the oestrogen receptor negative tumours and (ii) the oestrogen receptor rich tumours. Also for SBA the carcinomas with few

Table I—The relationship between the proportion of tumour cells binding specific lectins and steroid receptor status

	Many	PNA Mod.	Few	Many/Mod.	SBA Few	Neg.	Many	WGA Mod.	Few
Oestrogen receptor									
Negative	5	6	3	1	3	10	6	6	2
Poor	4	3	2	4	4	1	4	3	2
Rich	18	12	0	14	9	7	21	8	1
Progesterone receptor									
Negative	12	13	4	7	10	12	15	11	3
Positive	5	6	1	6	3	3	8	3	1

Growth of human breast cancer cells inhibited by a luteinizing hormone-releasing hormone agonist

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About one-third of human breast cancers require hormones for their continued growth¹, and endocrine ablation or anti-hormone therapy can cause regression of these tumours. As a consequence, ovariectomy in premenopausal women or administration of an anti-oestrogen (tamoxifen) in postmenopausal women represent major options for treatment of metastatic breast cancer. Alternatively, chronic administration of agonistic analogues of luteinizing hormone-releasing hormone (LHRH)² causes regression of mammary tumours in experimental animals³⁻⁶, and such treatment has shown promise in a small series of premenopausal women with advanced breast cancer⁷. It has been assumed that these results were achieved by suppressing the pituitary-ovarian axis, as the treatment causes a reduction in circulating levels of gonadal steroids similar to that produced by castration⁶⁻⁸. However, LHRH agonists can exert major effects on tissues other than the pituitary in animals^{9,10} and in the human¹¹⁻¹⁵. Such findings, coupled with reports of LHRH in human breast milk¹⁶ and immunohistochemical evidence for the presence of LHRH-like activity in some human breast tumours¹⁷, prompted us to test whether LHRH agonists could have direct antitumour effects. We now report major direct effects of LHRH and its agonists on the growth of breast tumour cells in culture.

For these studies we used the MCF-7 breast tumour cell line, the growth of which is responsive to sex steroids¹⁸⁻²⁰. In preliminary studies, addition of the LHRH agonist D-Ser-(Bu¹)⁶LHRH(1-9)-ethylamide (HOE 766; Hoechst) to MCF-7 cells every 2 or 3 days during culture inhibited cell growth, but this effect was not major and was inconsistent. However, when the LHRH agonist was added daily to the MCF-7 cells it caused major consistent suppression of cell growth in a dose-related manner (Fig. 1a). Culture of cells in the presence of concentrations of LHRH agonist in excess of 10^{-9} M resulted in a net decrease in cell numbers over the 4-day period of the experiment. These effects were highly reproducible (Table 1). It was also possible to elicit inhibitory effects on cell growth using native LHRH, although much higher concentrations were required than with the LHRH agonist (Fig. 1b). The major inhibitory effects of LHRH agonist on cell growth were blocked completely by addition of the LHRH antagonist [N-Ac-D-Nal(2)¹, D-pCl-Phe², D-Trp³, D-Har(Et)⁶, D-Ala¹⁰]LHRH, the combination of this peptide and the agonist having no greater effect than the

antagonist alone (Fig. 1c); the latter caused only minor but statistically significant suppression of cell growth.

The fact that the LHRH antagonist blocks the inhibitory effects of the LHRH agonist on the tumour cells suggests the involvement of specific receptors. Using whole cell preparations of the MCF-7 line, we were able to demonstrate specific binding of ¹²⁵I-labelled LHRH agonist (Fig. 2), for which LHRH showed minor but significant competition whilst thyrotropin releasing hormone (TRH) had no effect. The concentrations of LHRH agonist required for displacement (10^{-6} – 10^{-4} M) indicate that the binding sites are of apparently low affinity when compared with extrapituitary binding sites for LHRH agonists in the rat^{9,21}. However, the present observations are similar to those in other human extrapituitary tissues (ovary, testis and placenta) which also possess low-affinity binding sites for LHRH agonists^{13,22-24}. Moreover, in these tissues^{11,15,24-26}, as in the MCF-7 cells studied here, concentrations of LHRH agonist which are insufficient to displace the binding of radiolabelled tracer under receptor-assay conditions (10^{-9} M) are capable of eliciting clear-cut biological effects. This paradox is unexplained but it may be simply a reflection of the different time periods and temperatures used for assessment of the receptor-binding potency of LHRH agonists and that used for assessment of the biological potency. Alternatively it is possible that high-affinity binding sites for LHRH agonist are present in MCF-7 cells and these other tissues, but that they represent a small proportion of the total binding sites present. It is also noteworthy that, in the present studies, maximum binding of radiolabelled LHRH agonist was obtained after only 10 min incubation at 21 °C. Longer incubation (20–30 min) resulted in a marked decrease in binding (data not shown), presumably as a result of proteolytic degradation. Again, this is similar to findings for binding of LHRH agonist to the human ovary, testis and placenta^{13,22,24}. This presumed proteolytic activity associated with MCF-7 cells would also explain our inability to show consistent inhibitory effects of the LHRH agonist on cell growth unless the media were replenished with fresh LHRH agonist every 24 h.

To our knowledge, the present data represent the first unequivocal demonstration that LHRH is capable of exerting major direct effects on the growth of human breast cancer cells. The possibility that these effects represent an anti-steroidal action, as has been reported for LHRH agonists in other tissues^{27,28}, cannot be excluded at this stage, as the growth of the MCF-7 cell line may be stimulated by small amounts (10^{-11} M) of oestradiol²⁹ such as are present in the fetal calf serum included in these cultures.

At present, trials with LHRH agonists are being conducted in premenopausal women as a form of medical ovariectomy^{30,31}, but if direct effects on breast tumour cells are possible there is no reason why application of this therapy should be restricted to premenopausal patients. Indeed, treatment with LHRH agonist can produce objective responses in some postmenopausal women with breast cancer and inactive ovaries³², results which are consistent with direct antitumour effects of the LHRH agonist. However, as the present findings suggest that the LHRH agonist had to be replenished daily to be effective in inhibiting tumour cell growth, then conventionally used doses of LHRH

Table 1 Reproducibility of the inhibitory effects of LHRH agonist on cell numbers during culture of the MCF-7 line

Concentration of added LHRH agonist (M)	No. of experiments	Day of culture			
		1	2	3	4
0	(7)	1.35 ± 0.19	1.93 ± 0.39	2.49 ± 0.36	2.89 ± 0.39
10^{-9}	(5)	1.14 ± 0.06*	1.07 ± 0.13§	0.77 ± 0.11§	0.64 ± 0.15§
10^{-7}	(3)	0.96 ± 0.05‡	0.90 ± 0.14‡	0.58 ± 0.12§	0.39 ± 0.10§
10^{-6}	(2)	0.89 ± 0.10†	0.85 ± 0.04‡	0.51 ± 0.01§	0.15 ± 0.07§

Values are the mean ± s.d. and refer to the ratio of cell numbers on the day of collection to that (0.5×10^6) plated initially on day 0. Experimental conditions and statistical analysis were as described for Fig. 1.

* $P < 0.05$; † $P < 0.02$; ‡ $P < 0.01$; § $P < 0.001$; in comparison with respective control values (Student's *t*-test).

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DIRECT EFFECTS OF LHRH AND AGONISTS ON HUMAN BREAST CANCER CELLS

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INTRODUCTION

The growth of the human female breast is clearly under endocrine control. In the absence of stimulatory hormones, the breast remains rudimentary whilst major development normally only occurs at times of increased hormone production such as puberty and pregnancy. It is, therefore, not surprising that certain breast cancers should also require hormones for their continued growth. Whilst polypeptide hormones are capable of influencing events within breast cancers, (Dilley and Kister, 1975, Leung and Shiu, 1981) the most convincing evidence for the involvement of endocrine agents is for steroid hormones, particularly oestrogens. This includes the observations that, a) the growth of cell lines of human breast cancer may be stimulated by oestrogen both in culture (Butler et al, 1981, Benz et al, 1983) and in immunosuppressed animals (Shafie 1980, Seibert et al, 1983); b) the administration of oestrogen to breast cancer patients with metastatic deposits in bone may cause increased urinary calcium excretion due to accelerated tumour growth (Pearson et al, 1954); c) oestrogen deprivation therapy causes regression of breast cancers in about one-third of patients with advanced disease (Henderson and Canellos, 1980); d) these effects are invariably associated with tumours which possess receptors for oestrogen (Jensen et al, 1974, McGuire et al, 1975). Endocrine intervention therefore represents a major treatment option for advanced breast cancer especially against tumours which are rich in oestrogen receptors. In premenopausal women, therapy usually takes the form of castration which classically is achieved by surgical or radiological ablation of the ovaries. However, with the observation that chronic administration of agonist analogues of LHRH suppresses the pituitary-ovarian axis (Schally et al, 1970, Petrosa et al, 1980) and may reduce circulating oestrogen to castrate levels (Nicholson and Maynard, 1979, Klijn and de Jong, 1982), the concept has developed of using LHRH agonists in the treatment of breast cancer as a form of chemical ovariectomy (Corbin, 1982, Furr and Nicholson, 1982). Whilst clinical studies with LHRH agonists have largely been performed in premenopausal women (Klijn et al, 1984) there are reports of beneficial anti-tumour effects in postmenopausal patients who were without measurable ovarian function (Harvey et al, 1984). Such observations raise the likelihood of direct effects of LHRH agonists on breast tumours, a possibility supported by the increasing number of reports describing effects of LHRH agonists on extra pituitary tissues (Khodr and

Siler Khodr, 1978, Lamberts et al, 1982, Tureck et al, 1982, Tan and Rousseau, 1983).

The evidence will therefore be presented that LHRH agonists have direct inhibitory effects on the growth of breast cancer cells in culture and that tumour cells possess specific binding sites for LHRH and its agonist in addition to the data that indicate that LHRH agonists produce a therapeutic castration and thereby are of benefit to premenopausal women with advanced breast cancer.

LHRH agonists as therapy for premenopausal patients with advanced breast cancer

The use of chronic LHRH agonist treatment as a contraceptive to suppress follicular development and ovulation is well established (Bergquist et al, 1981, 1982a- Schmidt-Gollwitzer et al, 1981, 1982). The exact mechanism by which this is achieved is not fully elucidated but pituitary desensitization (Belchetz et al, 1978; Sandow et al, 1978; Bergquist et al 1982b) which reduces gonadotrophin drive to the ovaries, is primarily implicated. Chronic administration of LHRH agonists to premenopausal women therefore reduces circulating oestrogens to castrate levels, although this may take several months to achieve (Nicholson et al, in press). These observations drew attention to the possibility of using LHRH agonists to treat premenopausal women with oestrogen dependent breast cancer (Corbin, 1982, Furr and Nicholson, 1982). So that a consistent suppression of gonadal steroids may be maintained, higher doses of agonist are required than for contraception. This can be achieved via nasal spray (Hardt and Schmidt-Gollwitzer 1983) but daily injections or infusions result in more reliable suppression (Klijn and de Jong, 1984).

To date, only small numbers of patients with advanced breast cancer have been treated with LHRH agonists. Klijn and de Jong (1984) have used the LHRH agonist, Buserelin, in a variety of regimes involving parental administration followed by either intranasal or subcutaneous treatment alone or in combination with other endocrine agents. Of a total of twenty two women receiving agonist alone, four patients had an objective response and another four had stabilization of disease. Treatment was associated with anovulation in all patients although transient peaks of plasma oestradiol were detected in most. Beneficial effects of other LHRH agonists have also been documented. Nicholson et al (1985) reported responses in three of fifteen and Harvey et al (1984) in two of four premenopausal women with advanced breast cancer treated with Zoladex.

This overall response rate to LHRH agonists is similar to that which would have been obtained by ovariectomy and there is reason to believe that, in the majority of premenopausal women, the benefits of LHRH agonists were achieved by a castration-like effect. Thus, in general, patients with the best responses to LHRH agonist had complete endocrine castration and possessed oestrogen receptor-positive tumours whilst non-responders to LHRH agonists rarely responded to subsequent removal of their ovaries. Only single cases have been reported of (a) a response in a patient with persistent plasma oestrogen following LHRH agonist therapy (Klijn et al, 1984), (b) remission in an oestrogen receptor negative tumour (Klijn et al, 1984) and (c) response to surgical castration in a patient previously failing agonist therapy (Nicholson et al, 1985). Treatment with LHRH agonist is, however, unlikely to replace surgical removal of the ovaries as the primary endocrine therapy in premenopausal women with advanced breast cancer - at least at the present time. Although ovariectomy is irreversible, it is a surgical procedure with very little morbidity and, because of the age-related incidence of breast cancer (Thomas, 1983), the operation is usually

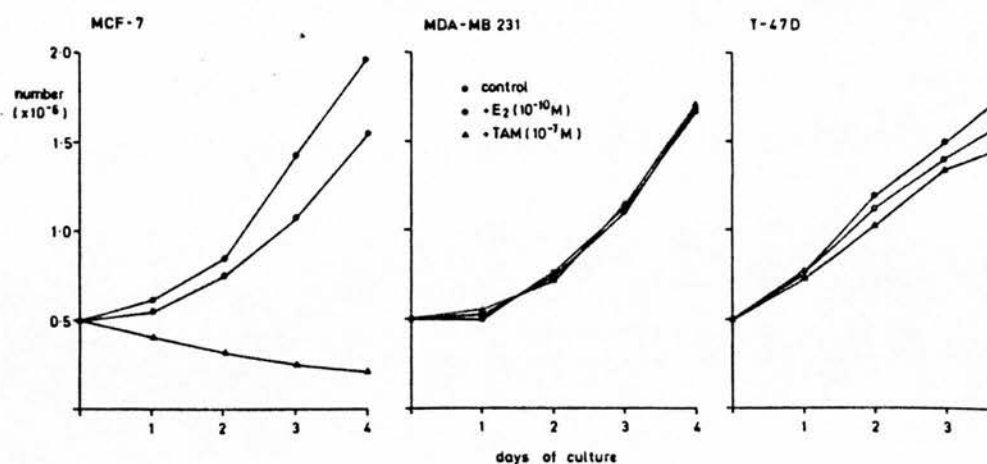


Fig. 1 The effects of oestradiol (E_2) and antioestrogen, tamoxifen (TAM) on the growth of human breast cancer cells in culture. Each point represents the mean value of triplicate systems.

To study effects on growth in culture, cells were grown at 37°C in Dulbecco's minimal essential medium containing 10% heat-inactivated foetal calf serum, under a humidified atmosphere of 5% CO_2 :95% air. Once growing in log phase, cells were collected and plated out in 60mm Petri dishes (0.5×10^6 cells in 4ml culture media). Sufficient dishes were set up so that each test system could be studied in triplicate at each time point. Hormones were added to the appropriate test systems after being dissolved in culture media. Cells were incubated for either 1, 2, 3 or 4 days, media being replenished each day. Growth was monitored by counting harvested cells in a haemocytometer after collection by trypsinization. Further experimental detail has already been published (Miller et al, 1985).

The effects of the LHRH agonist, buserelin, on the growth of MCF-7 cells is shown in Figure 2. All concentrations of the agonist other than 10^{-11} M caused significant ($p < 0.001$) inhibition of growth in comparison with control cells. These inhibitory effects were dose-related and concentrations of buserelin in excess of 10^{-9} M produced a net decrease in cell numbers after four days of culture. Levels in excess of 10^{-7} M agonist resulted in a progressive decrease in cell numbers over the whole test period. Inhibitory effects were highly reproducible and could be detected microscopically (see Plate) as early as day 2 of culture. Although cell numbers are decreased even after short exposure to LHRH agonist, the remaining cells appear viable and, after an initial delay, will show increased growth if cultured in media without LHRH agonist. The specificity of these inhibitory effects was studied by performing similar studies with native LHRH, the 3-10 fragment of LHRH and the LHRH antagonist, $\text{N-Ac-D-Na}(2)^1$, D-pCL-Phe^2 , D-Trp^3 , $\text{D-hArg}(\text{Et}_2)^6$, D-Ala^{10} LHRH. Native LHRH was also capable of inhibiting cell growth although much higher concentrations were required than with the LHRH agonist (Figure 3). The 3-10 fragment of LHRH did not significantly affect the growth of the MCF-7 cell line. The major inhibitory effects of LHRH agonist on cell growth were completely blocked by addition of the LHRH antagonist, the combination of the two peptides having no greater effect than the antagonist alone (Figure 4); the latter only caused minor but nevertheless significant suppression of cell growth. These results suggest that LHRH and its

As judged by the displacement of labelled LHRH agonist by unlabelled LHRH agonist, the MCF-7 cells contained binding sites with a low affinity ($K_d \sim 4 \times 10^{-5}M$) for the LHRH agonist (Figure 7). However, these binding sites showed a high degree of specificity in that only peptides which were LHRH-like competed for binding with ^{125}I -LHRH agonists. Thus, two LHRH antagonists ($\{Ac3-4 \text{ dehydro-Pro}^1, D\text{-pFPhe}^2, D\text{-Trp}^{3,6}\}LHRH$ and $\{N\text{-Ac-D-Nal}(2)^1, D\text{-pCl-Phe}^2, D\text{-Trp}^3, D\text{-hArg}(Et_2)^6, D\text{-Ala}^{10}\}LHRH$) both competed for binding with higher affinity ($K_d \sim 10^{-6}M$) than did the LHRH agonist whilst native LHRH competed very poorly ($K_d > 5 \times 10^{-4}$). Surprisingly, $^{3-10}LHRH$, which is completely devoid of any biological activity in-vitro, displaced binding of ^{125}I -LHRH agonist nearly as effectively as did the unlabelled agonist itself. Of the other peptides tested, at concentrations up to $1mM$, only bradykinin caused any displacement of binding and this was considered to be due to alteration of the pH of the incubation medium which contained a pH indicator.

Whilst these results demonstrate the presence on MCF-7 cells of specific binding sites for LHRH-like peptides, the low affinity of these sites for the LHRH agonist is puzzling in that biological effects of the same agonist on growth of the MCF-7 cells in-vitro was obtained with doses ($10^{-9}M$) far lower than the apparent affinity (K_d) of the D receptor ($4 \times 10^{-5}M$) for this peptide. We still have no explanation for this disparity, but it should be emphasized that it has been evident in every binding experiment performed. One possibility is that the MCF-7 cells contain two classes of LHRH-receptors, one of high affinity but low capacity and the other of low affinity and high capacity, with the former mediating the biological effects on growth. Unfortunately, because of the low numbers of LHRH-receptors present and other technical limitations it is not feasible to test directly this possibility.

It is equally intriguing that LHRH-receptors with relatively low affinity (K_d 's of 10^{-6} to $10^{-7}M$) for the LHRH agonist used in the present studies, have also been reported in other human extra-pituitary tissues such as the placenta (Currie et al, 1981) and corpus luteum (Popkin et al, 1983, Bramley et al, 1985). Moreover, in the placenta, these receptors have been shown to mediate biological effects of LHRH-like peptides (Belisle et al, 1984), and the gene for LHRH has been isolated from the human placenta (Seeburg and Adelman, 1984).

Therefore, it appears that several human extra-pituitary tissues contain LHRH-receptors which have a considerably lower affinity for LHRH agonists than do the respective extra-pituitary LHRH-receptors in the rat testis (Sharpe and Fraser, 1980) and ovary (Clayton et al, 1979). This may indicate that the human extra-pituitary LHRH-receptors are designed to detect a locally-produced LHRH-like peptide, the structure of which is different from native hypothalamic LHRH. In this respect it is of interest that LHRH has been reported in human breast milk (Amarant et al, 1982) and LHRH-like activity has been located immunohistochemically in certain ductal carcinomas of the breast (Seppala and Wahlstrom, 1980). Most recently, LHRH-receptors have been identified in a high percentage of ductal breast carcinomas (Eidne et al, 1985), although it is puzzling that these receptors were shown to have a relatively high affinity ($K_d \sim 10^{-8}M$) for an LHRH agonist, a finding which contrasts with the present findings in cultured breast tumour cells. The only major differences in technique used by Eidne et al (1985), when compared with the present studies, were the use of a membrane preparation and the use of an LHRH nonapeptide agonist with modifications at positions 6, 7 and 9 of the molecule, as opposed to the agonist used in our studies which had modifications only at positions 6 and 9. In preliminary studies using membrane preparations from breast cancer tissue, we have again found binding sites with low affinity ($\sim 10^{-5}M$) for our LHRH agonist (data not shown), which suggests that the important

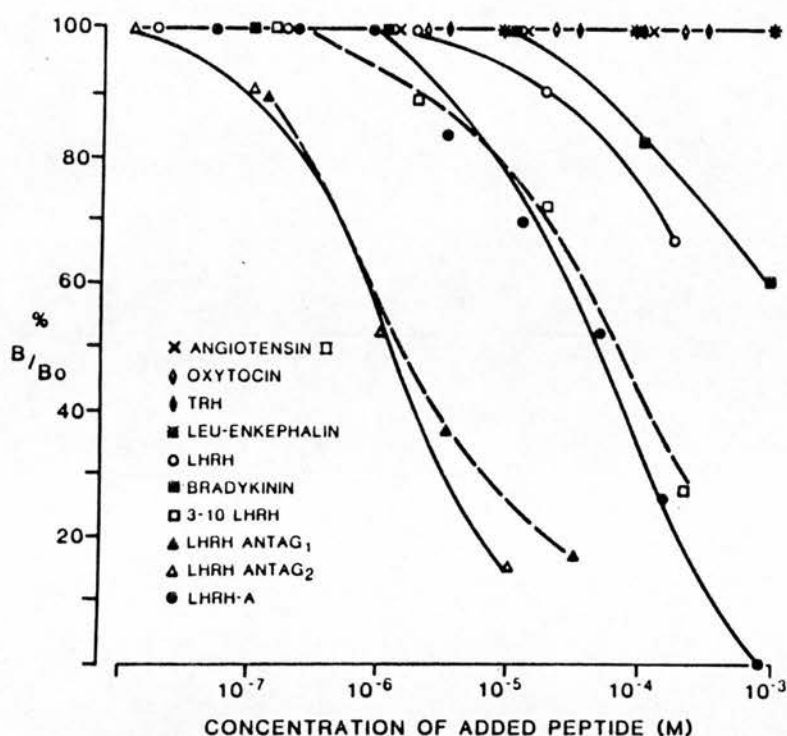


Fig. 7 Displacement of the binding of ^{125}I -labelled LHRH agonist to isolated MCF-7 cells by increasing concentrations of unlabelled LHRH agonist (LHRH-A), two LHRH antagonists (ANTAG₁ and ANTAG₂), LHRH and 3-10 LHRH as well as several other peptides. Non-specific binding was determined by addition of $7 \times 10^{-4}\text{M}$ unlabelled LHRH-A. Each point is the mean of triplicate incubations.

LHRH ANTAG₁ = (Ac3-4 dehydro-Pro¹, D-pFPhe²,
D-Trp^{3,6})LHRH

LHRH ANTAG₂ = (N-Ac-D-Nal(2)¹, D-pCl-Phe², D-Trp³,
D-hArg(Et₂)⁶, D-Ala¹⁰)LHRH

LHRH-A = (D-Ser-t-bu⁶, des-Gly-NH₂10)LHRH ethylamide

difference between our studies and those of Eidne et al (1985) is the use of an LHRH agonist with an additional modification at position 7.

Taken together, our findings and those of Eidne et al (1985) suggest that LHRH-like molecules may be capable of exerting direct effects on some human breast tumours, although what proportion of tumours might respond, and the nature of the response, remains to be determined. In our studies, we have tested for inhibitory effects of LHRH agonists on the growth of 3 breast tumour cell lines and only in the MCF-7 cells were major effects obtained; in the MDA-MB-231 cells no effects on growth were observed whilst in the T-47D line, only minor inhibition of cell growth was obtained (see Figure 5). However, a recent report has described major inhibitory

effects of an LHRH agonist on prolactin-stimulated growth of T-47D cells (Witzner and Benz, 1984), which perhaps suggests that LHRH exerts its inhibition only when growth of the cancer cells is stimulated, the nature of the stimulus being specific for different cell lines, i.e. oestrogen for MCF-7 cells and prolactin to T-47D cells. With respect to these findings, we have also identified low affinity binding sites for LHRH agonist in cells from the T-47D ($K_D \sim 6 \times 10^{-5}M$) and MDA-MB-231 ($K_D \sim 1.5 \times 10^{-4}M$) lines (data not shown).

SUMMARY

In premenopausal women with advanced breast cancer, treatment with LHRH agonists may cause a medical ovariectomy and thereby induce the regression of oestrogen-dependent tumours. However, there is good evidence from laboratory studies of breast cancer cells in culture and beneficial results of LHRH agonist therapy in postmenopausal women which suggest a more direct anti-tumour effect. The inhibitory action of LHRH and its analogues on cultured tumour cells appears to be mediated by a specific recognition mechanism and the presence of specific binding sites on breast cancer cells can be demonstrated. If the anti-tumour effects of LHRH agonist are to be fully exploited in clinical practice, future research should be directed towards elucidating the mechanism by which LHRH agonists inhibit tumour growth and to determining the optimal regime for administration of the agent.

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SECTION G

REVIEWS

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Hormones and Female Breast Cancer

Clinical Relevance

Andrew Patrick Forrest and William R. Miller

Breast cancer is the most common female cancer which is reported to now affect one in every 11 women in the United States.¹ It is a disease that typically disseminates early, but recurs late. Once invasive cancer is established, micrometastatic disease occurs quickly so that local treatment alone, traditionally believed to be the key stone of successful management, cannot hope to cure. Typically, the tumor is slow growing, so that many years may elapse before metastases become clinically evident. Such long-term follow-up studies, as that reported by Brinkley and Haybittle,² indicate that even 25 years following the initial treatment of breast cancer, women have an excess mortality from metastatic disease (Fig. 1). In their series of 704 patients treated in East Anglia between 1947-1950, statistical cure *i.e.*, the attainment of a group that is subject only to normal mortality rates, has not yet been demonstrated.

There would appear to be only three possibilities for improvement of this grave situation: (1) to employ effective systemic treatment to eliminate micrometastases at the time of initial treatment of invasive disease; (2) to detect the disease at a pre-invasive stage, *i.e.*, before micrometastatic spread has occurred; and (3) to prevent induction or promotion of the breast tumor by eliminating causative factors.

HORMONES

The growth of breast cancer, like that of cancer of the prostate, is influenced by hormones. This fact was unequivocally demonstrated by two surgeons: the first, George Beatson of Glasgow who, in 1896 reported that oophorectomy could induce remission of advanced disease in premenopausal patients;³ the second, Charles Huggins of Chicago, who demonstrated in 1945 that adrenalectomy had a similar effect in postmenopausal women.⁴ Evidence that the effect of castration on the progress of human breast cancer was due to deprivation of estrogenic hormones was first reported by Pearson and his colleagues in 1954, from the Memorial Sloan-Kettering Cancer Center in New York.⁵ They measured the daily excretion of calcium in the urine of six women with metastatic bone disease throughout several menstrual cycles, following oophorectomy and then after the administration of ethinyl-estradiol (Fig. 2). A cyclical

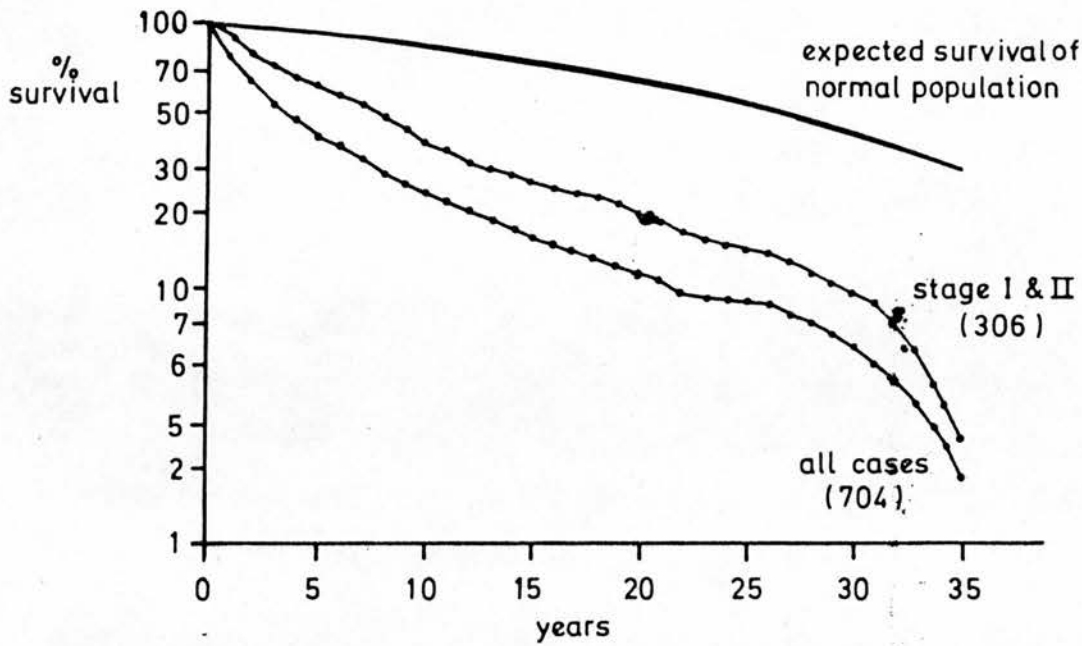


FIG. 1. Long-term follow-up of 704 patients with breast cancer after initial treatment in Cambridge, England. Redrawn from Brinkley DM, Haybittle JL.²

pattern of urinary calcium was evident during the menstrual cycle and was abolished by oophorectomy, when the level of urinary calcium dramatically fell. The subsequent administration of ethinyl-estradiol stimulated calcium secretion. These changes were believed to be due to the increase and reversal of bone breakdown as a result of removal and replacement of estrogen stimulation of the metastatic tumor.

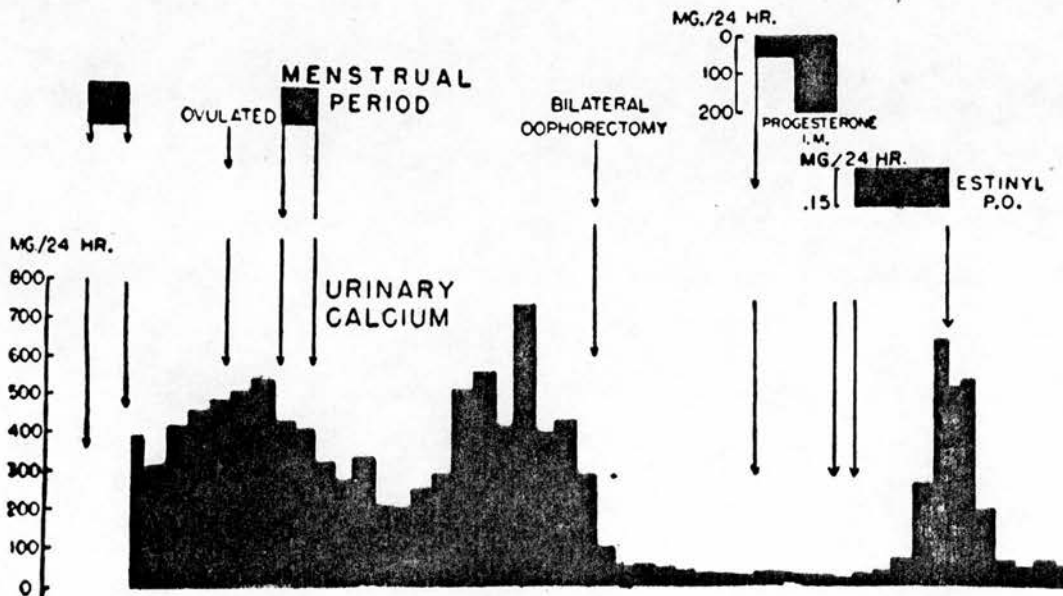


FIG. 2. Excretion of urinary calcium in postmenopausal women with breast cancer metastatic to bone before and after oophorectomy and following administration of ethinyl-estradiol. Reprinted with permission from Pearson OH *et al.*⁵

Further evidence for the involvement of estrogen in the growth of human breast cancer was provided by Jensen and colleagues,⁶ who demonstrated that the cytosol of approximately 70% of breast cancers contained high affinity binding proteins for estradiol similar to those present in the cells of other normal estrogen-sensitive tissues. This receptor mechanism is an essential step whereby estrogens exert their effect on nuclear events,⁷ cell division and growth. Work in several laboratories has shown that at least 90% of tumors that respond to hormonal treatment contain estrogen receptors, whereas cancers that do not possess this receptor mechanism rarely respond to hormonal measures, a fact of value in the management of patients with advanced disease.^{8,9}

Direct effects of estrogen on human breast cancer cells have been studied in the MCF-7 cell line, both in culture^{10,11} and following inoculation into immune-deprived (nude) mice.^{12,13} Thus, under appropriate culture conditions, concentrations of estrogen (as little as 10^{-11} molar), have been shown to promote cell growth¹⁴ (Fig. 3). Additionally, inoculates in nude mice will grow only in the presence of implants of estrogen.^{12,13} Recent work suggests that in the MCF-7 cell line, the effects of estrogen may be mediated through the autocrine action of induced growth factors.¹⁵

The rarity of the breast cancer in males, and the reduction in its incidence in women following castration early in life, suggests that estrogen is also an important factor for the development of the disease in humans. Evidence to implicate other hormones, *e.g.*, progesterone, testosterone, growth hormone, and prolactin in the initiation, promotion, or growth of human breast cancer, is less convincing.

Source of Estrogens

The major source of estrogens in premenopausal women is the ovaries, the cyclical secretion of which produces high circulating levels of estradiol and estrone at various periods of the menstrual cycle. Following the

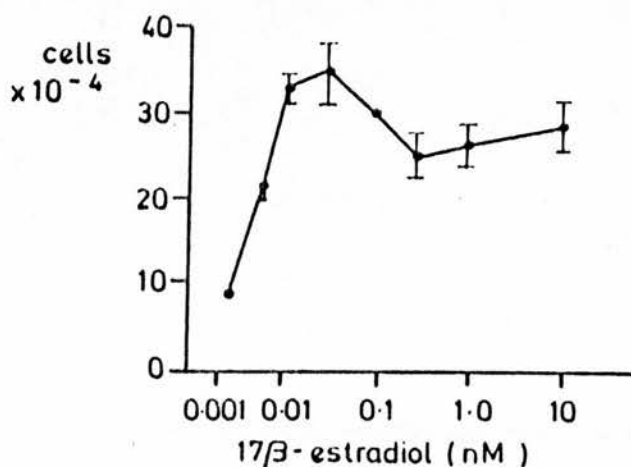


FIG. 3. The effect of estrogen on the growth of MCF-7 cells in culture. Redrawn from Benz C *et al.*¹⁴

menopause, circulating levels of estrogen are much reduced¹⁶⁻¹⁸ and they appear not to be derived either from the ovaries or, despite previous belief, the adrenals.^{19,20} In postmenopausal women, estrogens would appear to be formed by the peripheral conversion or aromatisation of precursor adrenal C19 steroids (dehydroepiandrosterone [DHA], its sulphate [DHA-S], and androstenedione).²¹⁻²³ There is good evidence that a major site for this activity is fat,²⁴ and this explains the correlation between the degree of obesity and the levels of circulating estrogen in postmenopausal women.²⁵ There is also good evidence for the local synthesis of estrogens within the breast both in fat²⁶ and also in the cancer itself.²⁷⁻²⁹ In our laboratory, one half of all breast cancers incubated *in vitro* with androgen precursors were found to synthesize estrogens.³⁰

The breast has an abundant supply of aromatizable C19 steroid precursors. Studies by our group,³¹ by Bradlow & co-workers in New York³² and by Angeli and associates in Turin,³³ have shown that human breast secretions, whether aspirated from the nipple or obtained by needle aspiration of breast cysts, contain remarkably high concentrations of DHA sulphate and other androgen conjugates (Fig. 4). The breast and its tumors,

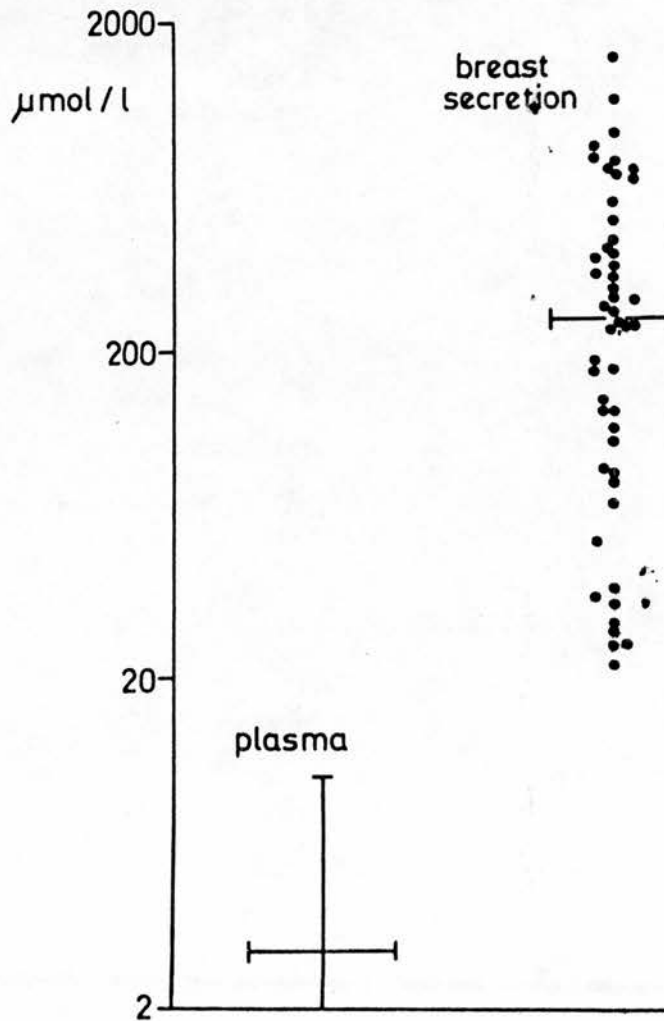


FIG. 4. Comparison of DHA-sulphate concentration on plasma of breast secretions of normal women.³¹

therefore, have the materials and mechanisms to form an effective factory for the local synthesis of estrogenic hormones.

THERAPY

The first method by which cure rates may be improved is the administration of effective systemic therapy to eradicate micrometastatic disease. Since the original studies of Fisher *et al.*³⁴ and Bonadonna *et al.*,³⁵ many controlled randomized trials have been reported in which it has been shown that chemotherapy, when administered as part of initial treatment, can prolong the interval to recurrent disease. Conclusions regarding effects on mortality have been less certain, possibly because the results of many trials are considered in isolation. An overview of data on mortality from all available trials involving over 10,000 randomized women was presented at a meeting convened jointly by the UK Breast Cancer Trials Co-ordinating Subcommittee and the Project on Controlled Therapeutic Trials of the UICC in London in October 1984, where it was reported that the administration of chemotherapy, particularly variants of the initial CMF (cyclophosphamide, methotrexate, 5-fluorouracil) regimen, significantly reduced short-term mortality (from a variable period of less than 1 to more than 5 years). In women first diagnosed after 50 years of age, reductions in mortality were only moderate (from 20% in control to 16%–18% in treated patients) but, in younger women, the effect was approximately twice as great.³⁶

Chemotherapy may have unpleasant side effects, and interest in hormonal manipulations has been rekindled by the report from Toronto, that when irradiation of the ovaries is combined with long-term steroid therapy (prednisone 7.5 mg daily) as part of the initial therapy of early breast cancer, survival in the premenopausal patient is significantly prolonged.³⁷ The development of a new range of pharmaceutical agents with potent anti-estrogenic effects, *e.g.*, tamoxifen (Fig. 5), aminoglutethimide (Fig. 6), and LH-RH agonists (Fig. 7), has further stimulated the use of endocrine treatment in early disease.

Tamoxifen

First tested in 1966 by Harper and Walpole,³⁸ tamoxifen, a synthetic triphenylethylene (Fig. 5), antagonizes the actions of estrogen by binding to the estrogen receptor protein but forming a complex which is ineffective.³⁹ Tamoxifen receptors have also been demonstrated in human breast cancer cytosols,⁴⁰ but their significance is uncertain. In MCF-7 human breast cancer cell line, tamoxifen inhibits growth, an effect which is reversed by the addition of estrogen.

The use of tamoxifen in postmenopausal women with advanced cancer of the breast is now well established, 30% to 40% obtaining benefit.⁴¹ As tamoxifen has few significant side effects, it has particular attractions for use as an adjuvant to primary therapy, and a number of controlled trials

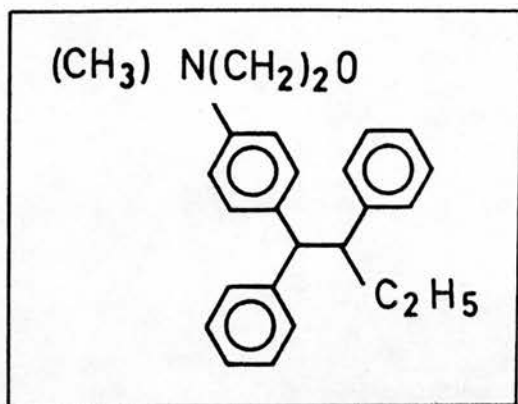


FIG. 5. Tamoxifen.

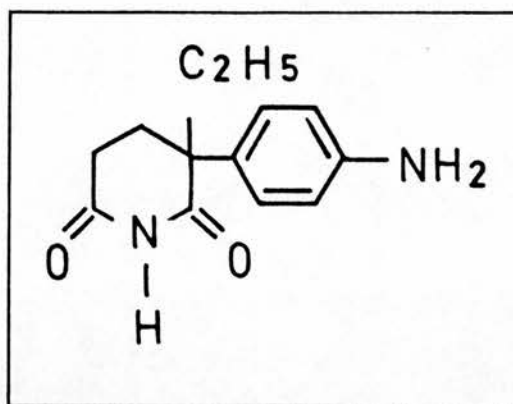


FIG. 6. Aminoglutethimide.



FIG. 7. LH-RH agonist: Buserelin.

comparing tamoxifen with no systemic therapy and tamoxifen plus chemotherapy, with chemotherapy alone, have now been reported. As with chemotherapy, prolongation of the disease-free interval has been repeatedly observed (Fig. 8) and firm evidence for reduction in mortality is now available. At the London overview,³⁶ which included 16,000 women admitted to controlled trials, a highly significant reduction in short-term mortality (less than 1 to more than 5 years of follow-up) was observed in women aged 50 years or older at diagnosis. This was similar in extent to that observed in women of this age with chemotherapy (see above).

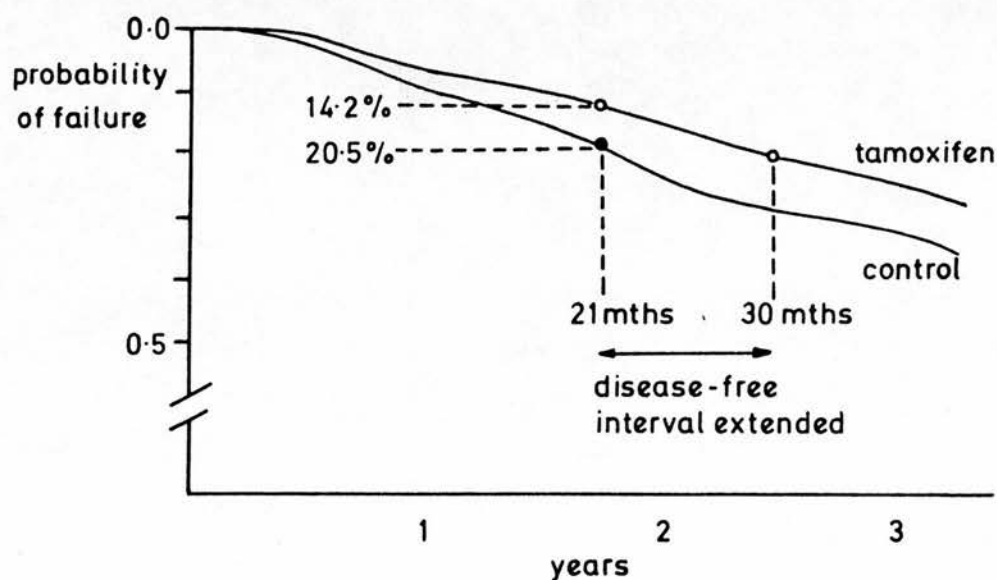


FIG. 8. The effect of adjuvant tamoxifen therapy on possibility of recurrence in 1285 women with primary cancer of the breast. Redrawn from Baum M *et al.*⁴²

It is important to know whether equal benefit can be achieved by the routine use of tamoxifen as first treatment for recurrent disease. This question is being addressed in Scottish centers in which tamoxifen therapy at the time of initial treatment is compared with tamoxifen given at the time of first recurrence.⁴³ In this trial, which includes over 1400 women, those receiving initial tamoxifen therapy do so for 5 years, when a secondary randomization is carried out to proceed with or discontinue the drug.

Aminoglutethimide

Initially developed for use as an anticonvulsant, aminoglutethimide, an analogue of glutethimide (a hypnotic) (Fig. 6), was withdrawn because of its suppressing effect on the function of the adrenal and thyroid glands. It was this property, of inhibiting adrenal synthesis of steroids, that led to its use combined with hydrocortisone as treatment of postmenopausal women with advanced breast cancer (medical adrenalectomy).⁴⁴ The action of aminoglutethimide on the adrenal is to inhibit the conversion of cholesterol to pregnenolone, which is the rate limiting step whereby steroid hormones are synthesized.⁴⁵ The enzyme inhibited is a cytochrome P450 complex, as are many other enzyme systems, including that of aromatization.⁴⁶ In postmenopausal women, this latter action would be directed against the peripheral synthesis of estrogens from C19-steroid precursors in fat and other tissues. We have demonstrated an anti-aromatase effect also in the breast by *in vitro* incubation of human breast cancer in the presence and absence of aminoglutethimide (Fig. 9).⁴⁷

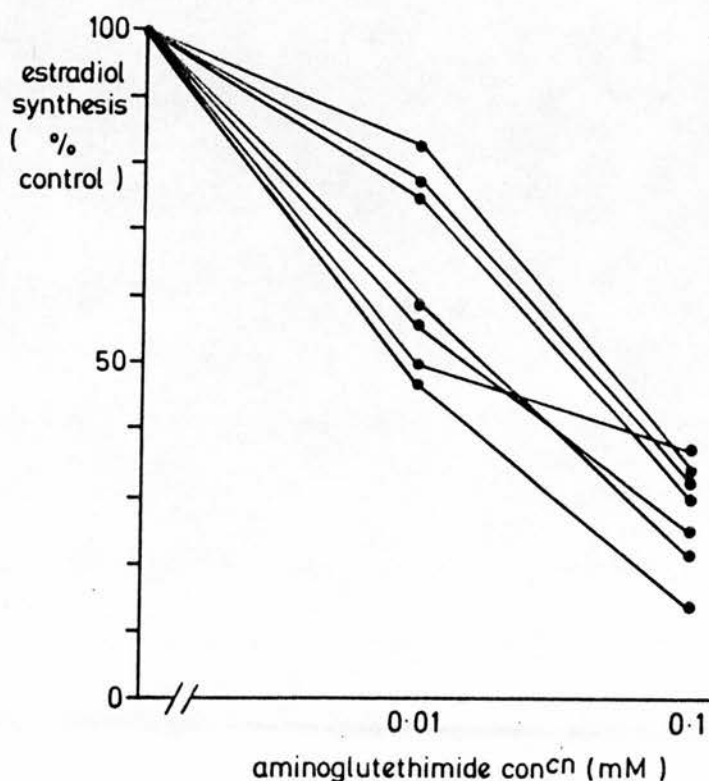


FIG. 9. Inhibition of aromatase activity: human breast cancer *in vitro* by aminoglutethimide.⁴⁷

The effect of aminoglutethimide in inducing remission of advanced breast cancer is more or less identical to that of adrenalectomy, and this has been confirmed in a controlled randomized trial comparing these two procedures.⁴⁸ The benefit achieved is similar to that of tamoxifen, but aminoglutethimide may induce a further response after initial tamoxifen therapy has failed.^{49,50}

Although side effects (gastrointestinal disturbance, lethargy and allergic manifestations) can be troublesome, a trial of aminoglutethimide as systemic treatment in early disease is underway.⁵¹ Preliminary reports suggest that the disease-free interval is prolonged, but survival data are not yet available.

Other more specific and more potent inhibitors of the aromatase system are being developed. These include 4-hydroxyandrostenedione,⁵² which shortly will be available for clinical trial.

LH-RH Agonists

Many of the synthetic analogues of luteinizing hormone-releasing hormone (LH-RH) have agonistic effects (Fig. 7). Their action is similar to that of pharmacologic doses of the natural hormone. After an initial surge in gonadotrophin release, the pituitary is desensitized to further stimulation so that secretion of LH and FSH is blocked.⁵³ The chronic effect in premenopausal women is to produce a "medical oophorectomy." The LH-RH agonist buserelin has also been shown in our laboratories to have a direct effect on the growth of the MCF-7 cell line in culture (Fig. 10).⁵⁴

A disadvantage of the initial preparations of these agonists was the need for continuous intravenous or frequent intranasal administration.

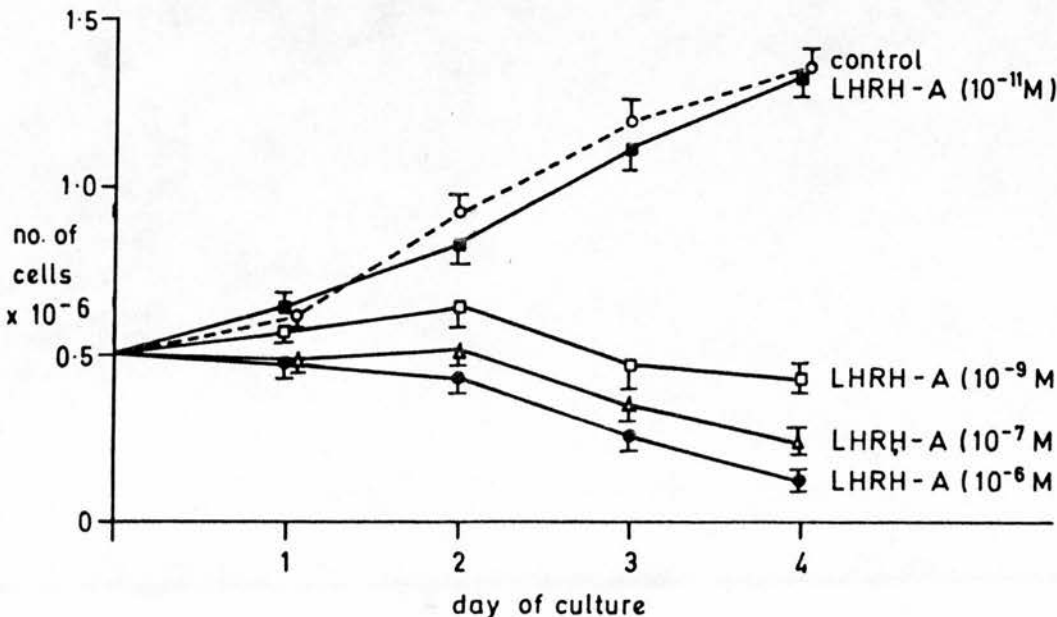


FIG. 10. Effect of LH-RH agonist on growth of MCF-cells in culture.⁵⁴

A long-acting depot preparation has now been developed. The use of this agent in patients with breast cancer is still at a preliminary stage,⁵⁵ but studies have been reported in male patients with advanced prostatic cancer.⁵⁶ Circulating levels of testosterone are suppressed, this being associated with clinical remission of the disease. This agent is clearly of considerable interest in the management of premenopausal women with breast cancer.

Prediction

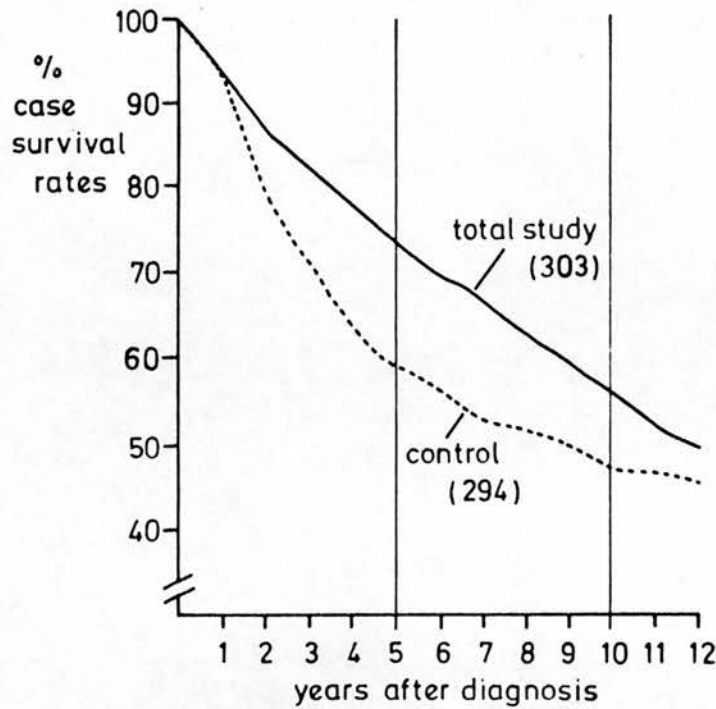
As only a proportion of patients have tumors that are sensitive to hormonal influences, the therapeutic effect of adjuvant therapy in those which do may be very great indeed. As indicated above, assays of estrogen receptor activity in a tumor can aid the prediction of response of patients with advanced breast cancer to endocrine therapy. Additional discrimination has been reported by simultaneous assays of receptor for progesterone receptor activity, replenishment of which is apparently dependent on an intact and functioning estrogen receptor system. Evidence is beginning to accrue that these assays may be of value in predicting which patients are most likely to gain benefit from adjuvant systemic therapy.⁵⁷⁻⁵⁹ Detailed studies of receptor activity and other possible indices of tumour sensitivity such as aromatase activity are clearly required.

EARLY DIAGNOSIS

There is firm evidence that the early detection of breast cancer through regular screening by clinical examination and mammography influences the mortality of the disease beneficially. This has come from the randomized study carried out in New York, in which 30,000 women age 50 to 69 years were offered annual clinical and mammographic screening on four occasions; 30,000 women, not offered screening, acted as controls.⁶⁰ Over an initial 5-year period, 303 cancers were detected in the screened group and 284 in the controls. Case fatality rates proved significantly better in the women in the group offer screening (Fig. 11), an effect most pronounced in those whose cancer had been detected by screening and particularly when the lesion was only evident on mammography (Fig. 12). Because the age-specific mortality from breast cancer in the population offered screening has also proved to be significantly less than in the control group over a period of 14 years, lead-time bias cannot account for this difference. Several controlled randomized trials are also in progress in Sweden, Canada, and in Edinburgh, Scotland, but case fatality and mortality statistics are not yet available.

Mortality apart, cancers detected through screening are smaller in size and less likely to have metastasized to the regional lymph nodes than those which are discovered spontaneously. This has the advantage of allowing more conservative local therapy with preservation of the breast.

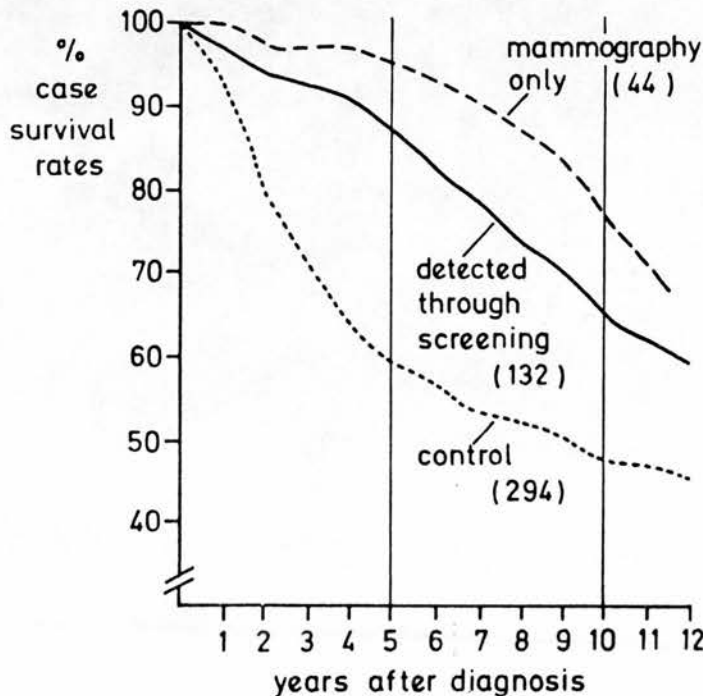
FIG. 11. Case fatality rates in 303 cancers in populations of women offered screening for breast cancer compared to 294 cancers arising in the control population. Redrawn from Shapiro *et al.*⁶⁰



In the United States, regular clinical and mammographic examination of the breast is recommended for all women older than age 50 years.⁶¹ This is expensive, and simpler methods of presymptomatic detection, *e.g.* by single oblique mammograms, are under trial elsewhere.⁶² The selective screening of those known to be at risk could have many advantages.

Factors known to influence the risk of breast cancer include age, a family history of breast cancer, and previous benign breast disease. En-

FIG. 12. Comparison of case fatality rates in women whose cancer was detected through screening with those in the control population. Redrawn from Shapiro *et al.*⁶⁰



vironmental factors are also important, and include social class and the Western way of life: dietary fat and body weight are considered by some to be most relevant.^{63,64} Other more direct hormonal influences have also been shown to bear on breast cancer risk.^{65,66} The ablation of ovarian function early in life reduces the incidence of breast cancer,⁶⁷ whereas prolongation of active menstrual life increases this. Nulliparous women are more at risk of developing the disease than are multiparous, and the greater the number of pregnancies the more is its incidence reduced.⁶⁶ This is largely, but not entirely, explained by the younger age at first full-term pregnancy in those with multiple births, an association first brought out in an international study reported by MacMahon and colleagues.⁶⁸ A recent case-control study has indicated that US women having their first full-term pregnancy after the age of 30 years have a four- to five-fold excess risk compared to those having their first birth before age 18 years.⁶⁹

If hormonal factors are of importance as determinants of risk, it would be expected that exogenously administered hormones would influence the risk of breast cancer. Three areas have provoked intense investigation: (1) the administration of high doses of stilbestrol (up to 15 g/pregnancy) during pregnancy; (2) use of oral contraceptives; and (3) hormone replacement therapy in postmenopausal women. To date, the evidence remains controversial (for recent review see reference 70).

Of recent interest was the observation by Pike and colleagues^{71,72} that the prolonged use of oral contraceptives before the age of 25 years was associated with an increased risk of breast cancer. This finding has been confirmed by a study from Oxford.⁷³ Although it was suggested that the increased risk was particularly associated with oral contraceptives of high progestogen potency, the classification of progestogenic activity (based on delay in menses) is not valid for the effect of progesterone on the breast.⁷⁴ Other case-control and cohort studies in young women are now underway to examine this finding further.

The search for an underlying endocrine abnormality associated with an increased risk from breast cancer is clearly worthwhile. Numerous case-control studies have been carried out, in which assays of plasma and urinary hormones in women with established disease have been compared with normal controls. In general, the results are conflicting. Such studies have a major drawback: the hormone milieu may radically change between initial induction of the tumor and clinically evident disease. Prospective studies, in which the endocrine profile is established in normal women who are subsequently followed up to determine whether they develop breast cancer, are of greater relevance. Examples are the studies of Bulbrook and associates in the Island of Guernsey^{75,76} and DeWaard and co-workers in Utrecht.⁷⁷ Such prospective studies are complex and expensive. Comparisons of hormone levels in women known, for other reasons, to be at high or low risk are more easy to perform.

Examples include comparisons of Western and Asiatic women,^{78,79} women with and without a strong family history,⁸⁰ and nulliparous and parous women.⁸¹ Various differences have been described, some of the most important of which are summarized in Table 1.

The results of assays of circulating hormones are difficult to interpret. Differences are small and often conflicting. There are sampling problems (hormone levels do not remain static over a 24-hour period), and when differences are reported, they are often of statistical import for groups rather than discriminatory in individual patients. The advent of salivary assays, which do not require repeated intravenous sampling, may allow some of these difficulties to be resolved.

Of greatest interest are those studies which may monitor the supply of hormones actually available to the breast, *e.g.*, assays of free circulating estrogen.⁸⁴ This can also be studied by assays of the hormonal composition of human breast secretions, but information on estrogen content of such fluids is scanty.

The application of risk factors and hormonal assays to the detection of women at risk from breast cancer has not proved fruitful. These have

TABLE 1. Examples of Hormonal Changes Described in Women at Varying Risk From the Development of Breast Cancer

Hormones	Population studied	Results	Reference
Urinary androgens	Women subsequently developing breast cancer <i>versus</i> those remaining disease-free	Lower etiocholanolone in cancer group	75, 76
	Japanese <i>versus</i> British women	No difference Lower etiocholanolone in Japanese	77 78
Urinary estrogens	Asian <i>versus</i> Western women	Estriol ratio higher in Asians	79
	Parous <i>versus</i> nulliparous	Estriol ratio higher in parous	81
	Parous <i>versus</i> nulliparous	No difference	82
	No family history <i>versus</i> strong history	No difference	80
Total plasma estrogens	No family history <i>versus</i> strong history	Higher estrogens in strong history	83
		No difference	80
Free plasma estrogen	Women subsequently developing breast cancer <i>versus</i> those remaining disease-free	Higher free estradiol in breast cancer group	84
16 α hydroxylation	No family history <i>versus</i> strong history	Higher in strong history	85
Nocturnal plasma prolactin	Nulliparous <i>versus</i> parous	Lower in parous	86
Plasma progesterone	Benign disease <i>versus</i> normal women	Lower in benign disease	87
Salivary progesterone	Benign disease <i>versus</i> normal women	No difference	88
		No difference	89

been tested in combination in the HIP study of New York, the Guernsey study and the BCDDP project, but the sensitivity and specificity is too low to allow the definition of a sufficiently small proportion of women which contains the majority of cancers.⁹⁰⁻⁹² For example, using a logistic analysis including age at menarche, family history, age at first birth, and etiocholanolone excretion, Farewell was able to identify only 82% of cancer cases in 55% of the population (or 47% of the cancers in 17% of the population).⁹⁰

PREVENTION

From these considerations, it seems unlikely that hormonal assays will permit the definition of a pattern of abnormality which will identify women at risk with sufficient precision to be useful in a breast cancer screening program. This is disappointing as, were it possible to detect "risk" patterns of hormone levels, the way would also be paved for intervention studies that could lower the incidence of the disease.

A good example is the suggestion that luteal phase insufficiency, *i.e.*, inadequate progesterone secretion in the second half of the menstrual cycle, may, by allowing unopposed estrogen action on the breast, increase risk (Fig. 13). Were this so, the defect could readily be corrected by progesterone administration. Unfortunately this suggestion is not supported by recent evidence from the use of serum and of salivary assays to document circulating progesterone levels in groups of women with and without breast disease during whole menstrual cycles (Fig. 14).^{88,89} Before corrective therapy can be applied, there must be uncontroversial evidence of a reproducible and correctable abnormality, and it is there that the

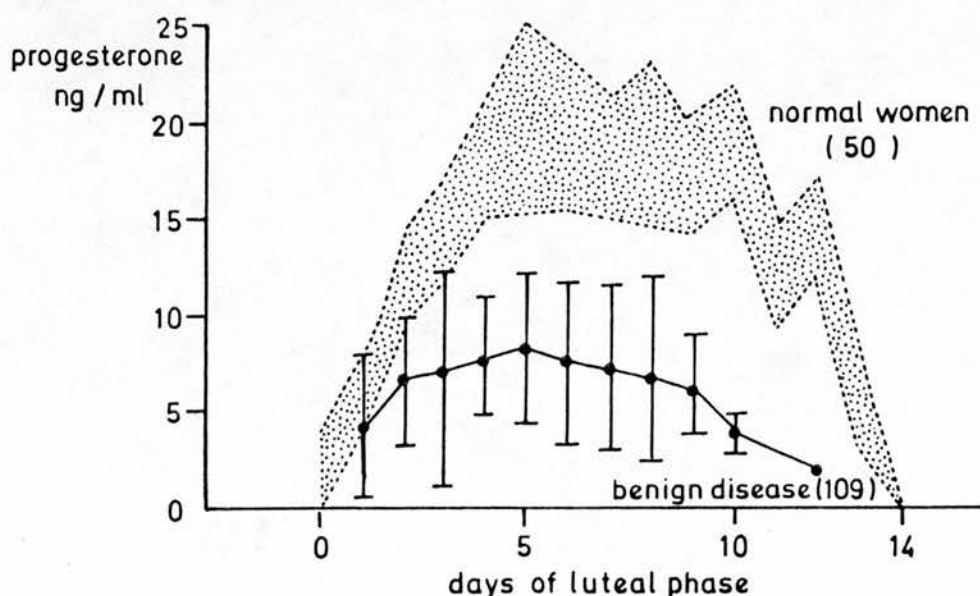


FIG. 13. Plasma progesterone values: 109 patients with breast disease and 50 control women during days 1-12 of the thermal plateau. Reproduced with permission from Mauvais-Jarvis P *et al.*⁸⁷

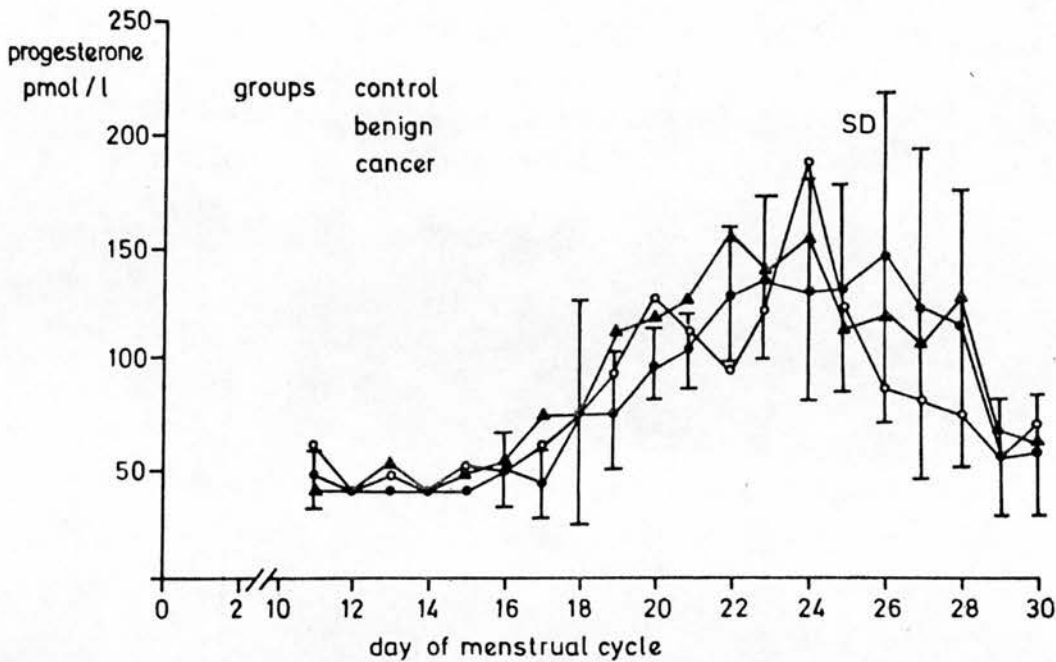


FIG. 14. Circatrigintan variations in salivary progesterone in premenopausal women. Reproduced with permission from Read GF *et al.*⁸⁹

problem lies. We are still a long way from using hormonal manipulations to prevent breast cancer.

CONCLUSIONS

It is concluded that knowledge of hormonal aspects of breast cancer has not proven to have immediate clinical relevance, either in the early diagnosis of the disease or its prevention. However, the development of new therapeutic agents which influence the endocrine system now offers hope of the better control of established disease. Breast cancer starts in the epithelium of the lobule and its terminal duct, and it is the supply of estrogen and possibly other hormones to that composite unit (terminal duct lobular unit) which is likely to prove of greatest significance. For further progress, hormonal interactions within the breast need to be studied.

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[Discussion of this paper is included in the discussion following the next presentation.—Ed.]

OESTROGENS, PROGESTOGENS AND THE BREAST

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INTRODUCTION

It is common knowledge that hormones control normal breast development in the human female. Growth of the breast mostly occurs at puberty and during pregnancy and, conversely, involutionary changes take place after pregnancy and the menopause. Hormone excess or imbalance is also implicated in the aetiology of breast abnormalities including cancer. Despite this, the parts played by individual hormones in these processes are ill-defined. This chapter will examine the roles of the ovarian steroids, oestrogen and progestogen, by considering (a) their involvement in the development of normal breast; (b) the histological appearance and biochemical activity of the breast in the absence of ovarian production of these steroids, i.e. after the menopause or castration; (c) the effects of exogenous administration of oestrogen and progestogen, particularly in postmenopausal women and (d) the evidence that risk of breast cancer is changed by prolonged stimulation with oestrogen alone or in combination with progestogen as might occur following late natural menopause or replacement hormone therapy.

HORMONES AND BREAST DEVELOPMENT

Development of the Adult Breast

The breast is a relatively quiescent organ until puberty. At this time, which may precede first menstruation by several years, discs of stromal connective tissue develop underneath the nipple and areola. Within these discs, lactiferous ducts lengthen and branch to open at the nipple which itself becomes everted. Simultaneously, lobular buds develop although the lobules themselves are not completely formed until ovulatory menstrual cycles occur. Mature breast lobules are the functional units of the adult breast (Figure 1a) and thousands may exist in each breast. The epithelial (or

parenchymal) component comprises only a small proportion of the total breast volume and the breast is mostly composed of supporting fibrous tissue and fat.

Although the adult non-lactating breast produces limited amounts of secretion which appear to be reabsorbed during transit through the duct system it is only during pregnancy and lactation that the breast achieves full secretory status and all lobules fully develop. Both ductal and glandular elements of the breast proliferate greatly so that by the end of pregnancy, the breast is composed of a compact mass of lobules each containing multiple alveoli lined by secretory cells (Figure 1b). These produce colostrum during pregnancy and milk during lactation.

Hormone Involvement

Major breast development coincides with puberty and pregnancy. These are times at which the output of many hormones is dramatically increased. At puberty, hormones of pituitary origin increase in addition to ovarian production of oestrogen and progesterone. Similarly, during pregnancy, the foetal-placental unit not only secretes additional oestrogen and progesterone but is also responsible for the production of polypeptides with properties similar to those of growth hormone, prolactin and gonadotrophins. The roles of individual hormones are not readily defined and breast development probably requires synergistic cooperation between a multiplicity of hormones (Figure 2). The most convincing evidence for this comes from studies in experimental animals. Thus, the response of mammary tissue to hormones administered singly or in combination to rats in which endogenous hormones were reduced by oophorectomy, adrenalectomy and hypophysectomy showed that (a) the minimal requirements for ductal

growth were oestrogen, corticosteroid and growth hormone; (b) prolactin induced some development of lobules and alveoli; (c) full breast development similar to that seen in late pregnancy required at least oestrogen, progestogen, prolactin and growth hormone (Lyons et al. 1958). These observations are compatible with the hormonal changes and breast responses that occur naturally in women during puberty and pregnancy. Thus, spontaneous breast development does not occur in adolescent girls lacking oestrogen but oestrogen induced growth is dependent upon at least the simultaneous presence of gonadotrophins and cortisol (Perzelan et al. 1982). Similarly synergistic effects have also been noted between oestrogen and prolactin (Goldzieher, 1958).

Actions of oestrogen and progestogen

Because of synergism and other interactions, no individual endocrine agent can be considered in isolation. Nevertheless certain generalisations may be made about the effects of oestrogen and progestogen on the breast.

First, the major actions of oestrogen appear to be on the ductal system in the breast. In most animal species, oestrogen administration promotes proliferation of ductal epithelium although acinar development may also be stimulated, particularly with high concentrations (Bonser et al. 1961, Bassler 1970). Whilst data from experimental animals are not necessarily applicable to humans, histological examination of breasts from women receiving oestrogen therapy suggest that similar changes are induced in the human breast (Haagensen 1971). Differential sensitivity to oestrogen may also be demonstrated in explants of normal human breast growing in culture, larger ducts being more responsive than either intralobular ducts or the lobules (van Bogaert 1978).

In contrast to oestrogen, the actions of progesterone appear to be directed more towards lobulo-alveolar development. Thus administration of progesterone, especially in combination with oestrogen, to immature animals produces pronounced effects on acinar growth (Bonser et al. 1961, Bassler 1970). The sequence of events occurring within the human breast at puberty also suggest that the primary action of progestogen is to stimulate lobular development. Thus, lobulo-alveolar structures do not appear until the onset of ovulatory cycles in which progesterone is secreted from a functional corpus luteum (Bonser et al. 1961). In vitro studies are also confirmatory. Progesterone stimulates DNA synthesis in intralobular ducts from whose end-buds lobular-alveolar structures arise, established lobules and ducts being much less sensitive (van Bogaert, 1978). However, effects of progesterone on the ductal system cannot totally be excluded and DNA synthesis in ductal and ductular epithelium has been reported to be higher in the luteal phase of the menstrual cycle compared with the follicular phase (Meyer 1977).

In terms of secretory activity within the resting breast, as measured by nipple aspirates, we could find no difference between the phases of the menstrual cycle. However, in parous women oral contraceptives significantly reduced the incidence of breast secretions (Table 1), this effect being particularly associated with combined hormone pills. Interestingly, oral contraceptives did not reduce the frequency with which breast secretions were obtained from nulliparous women who, as a group, yielded secretions significantly less than parous women. These observations emphasise the complex interplay between oestrogen, progestogen and breast development and illustrate that (1) there is synergism (but little evidence of antagonism) between the hormones; (2) separate elements of the breast

have different hormone sensitivity; (3) differing hormone sensitivity may be displayed by the same elements of the breast (a) at different developmental periods, e.g. developing versus established lobules and (b) before and after pregnancy. As a result it can be predicted that, following the menopause, there will be great variation in atrophic changes between different parts of the breasts and different individuals and that the role of individual hormones in the development of breast abnormalities will be equally difficult to decipher.

THE POSTMENOPAUSAL BREAST

Involution

With the decline of ovarian function, breast involution occurs. The process may begin before cessation of menses, a preclimacteric-phase occurring between 35 and 45 years of age (Vorherr 1974). This involves a decrease in glandular epithelium with some involution of acinar and lobular tissue (Figure 3a). In the post-climacteric phase, there is a much more significant reduction of glandular tissue with a concomitant increase in fat deposition and a relative predominance of connective tissue (Figure 3b and c). The loss of lobular and alveolar structures is most striking and sometimes only the collecting ducts and microcystic remnants (Figure 3d) remain to mark the site of a lobule. Eventually the ducts also degenerate leaving only small islands of atrophic epithelial parenchyma surrounded by connective tissue and fat. The changes in relative proportions of the main elements are shown in Figure 4. Whilst glandular tissue might constitute about one third of the mature breast, after the menopause it rapidly diminishes and may represent only 5% of the gland in senility. In contrast the proportion of fat increases with age and represents the largest component of the postmenopausal breast. Involution is by

no means a uniform process. Frequently, one part of the breast may have lost all its lobules whilst another part still retains a normal lobular pattern. It is therefore not unusual to see numerous and well-preserved lobules in women of advanced age. The presence of persistent lobules (Figure 5a and b) in postmenopausal women may be associated with increased risk of breast cancer, being found more often in mastectomy specimens (for breast cancer) than in breasts at routine autopsy (Wellings, Jensen, and Marcus, 1975).

Secretory and Biochemical Activity

Although its glandular component is much reduced, the postmenopausal breast is not a completely inert structure. As is shown in Table 1 it is still possible to obtain nipple secretions from breasts of about one third of postmenopausal women. This clearly indicates that the remaining glandular elements of the breast are functional. Furthermore, parenchyma, stroma and adipose tissue from postmenopausal breasts are all able to metabolise steroid hormones including progestogens, androgens and oestrogens (Miller 1986). Pathways of activation and deactivation exist. One interesting route of metabolism is the conversion of oestrone sulphate which is the major, albeit inactive, circulating oestrogen into the most potent naturally-occurring oestrogen, oestradiol. Additionally, breast fat, a major constituent of the breast after the menopause, is able to synthesise oestrogen from androgen precursors (Berenek et al. 1984). This activity is not uniformly distributed throughout the breast and, for example, enhanced oestrogen synthesis is associated with fat from areas of breasts containing cancer. A localized phenomenon of this type could account for the focal nature of persistent lobules in postmenopausal breasts. Local biosynthesis of active hormones assumes

greater importance after the menopause once circulating ovarian-derived hormones diminish and may account for the high concentrations of both androgens and oestrogens within normal postmenopausal breasts (van Landeghem et al. 1985 a, b). These levels are not significantly different from those in premenopausal breast and are much higher than circulating postmenopausal concentrations.

Hormone Replacement Therapy in Postmenopausal Women

Little information is available on the effects of hormone therapy on the breasts of postmenopausal women and most is anecdotal. The single largest study is that of Huseby and Thomas (1954) who looked for changes in the normal atrophic breast tissue of patients with advanced breast cancer who were at least 5 years past their menopause and who were being treated with large doses of oestrogenic hormones. This type of study has limitations in terms of the "normality" of the breast tissue, the possibility that the presence of cancer or other previous therapies may have influenced the breast and the pharmacological doses of hormones administered. Of the 36 patients examined, breast tissue from 34 showed evidence of epithelial stimulation with increased numbers of small ducts, interlobular connective tissue and formation of new lobules. Whilst these changes are the reverse of the normal atrophic processes occurring at the menopause, only in one instance had all the breast tissue been "restored" to the premenopausal state. In the remainder, the histological pattern was distinctly abnormal. Such consistent changes have not been noted by others. Foote and Stewart (1945) could identify proliferative changes in only one of four breast specimens from postmenopausal or castrate women receiving oestrogen and, in a series of only 3 cases, Auchincloss and Haagensen (1940) found merely dilation of ducts in one specimen although the others had evidence of

marked epithelial stimulation with numerous large gland fields. In summary, therefore, although responses vary between individuals, administration of oestrogen in high doses to postmenopausal women tends to reverse the process of breast involution. The effects of progestogens either alone or in combination with oestrogen have not been documented.

OESTROGEN, PROGESTOGEN AND BREAST CANCER

The aetiology of breast cancer has a strong hormonal component (Table 2). In view of their proliferative properties within normal breast, it is understandable that oestrogen and progestogen should be implicated. The last section of this chapter will therefore present the aetiological evidence linking hormone excess or imbalance to increased risk of breast cancer and the possible mechanism by which oestrogen and progestogen may promote the development of this malignancy.

Aetiological Evidence

In humans, the single greatest risk factor for breast cancer is being a woman. The incidence in males is 1% of that in females, although administration of oestrogen, whether for sexual problems or for cancer of the prostate, increases the risk. In women, breast cancer does not occur before puberty and is rare before 25 years; thereafter incidence increases gradually with age until the menopause at which it decreases slightly before increasing again with advancing years (De Waard et al. 1964). There are also other factors which point to the involvement of hormones in cancer risk.

Thus an extended reproductive life whether by early menarche or late menopause increases a woman's risk of breast cancer (MacMahon et

al. 1975). The data for age at menopause are particularly impressive and it is estimated that women with a menopause at 55 years or older have twice the risk as those whose menopause occurred naturally before 45 years of age (Trichopoulos et al. 1972). Conversely, artificially induced menopause, particularly early in life protects against breast cancer (Feinleib 1968). Overall, surgical menopause is associated with a 40% reduction in risk and oophorectomy before the age of 35 years decreases the risk to one third of that in women with a natural menopause (Trichopoulos et al. 1972). These statistics have interesting implications suggesting that (1) ovarian activity plays a part in the development of at least 2/3 of breast cancers occurring in women undergoing a natural menopause and (2) as the breasts of women at 35 years have already been exposed to over half the normal period of ovarian activity, it is not simple exposure to ovarian hormones that causes breast cancer. The protection of oophorectomy and hazard of late menopause appear to be lifelong as is reflected by the reduced incidence of induced menopause (Lilienfield 1956) and the later age at natural menopause (by 1-2 years) (Haagensen 1971) of breast cancer patients compared with the general population.

Whilst it is generally agreed that a prolonged reproductive life increases risk of breast cancer, there is no consensus as to why this should be so. Indeed, two diametrically opposing theories have appeared. In one hypothesis (Henderson et al. 1985), it is suggested that increased risk results from the cumulative effects of regular cyclic stimulation to which the breast is exposed. Oestrogen and progestogen act in concert to promote proliferation of cells susceptible to carcinogenic agents. Proponents of this hypothesis cite, as supportive evidence, the protective action of pregnancy (MacMahon et al. 1973), especially early in life, which breaks up regular cyclicity. The second but opposite theory is based on

"oestrogen windows" (Korenman 1980) and postulates that, at each end of reproductive life, menstrual cycles are irregular and there are major periods of anovulation during which the breast is subject to oestrogens "unopposed" by progestogens. Certainly, prior to the menopause there is a variable period of time (as long as 8 years) during which menstrual cycles are irregular (Treloar et al. 1967). Longer cycles become more prevalent and these appear to be anovulatory (Sherman et al. 1976). There is also a suggestion that women with a late menopause have longer menstrual cycles than women with an early menopause (Wallace et al. 1978) and are therefore more likely to be exposed to more anovulatory cycles. Since anovulation is one of the major reasons for nulliparity, the increased risk of nulliparous women to breast cancer has been used to support the luteal inadequacy hypothesis. Indeed, it has been reported that infertile women with endogenous progesterone deficiency have over a five fold greater risk of breast cancer compared with those infertile for other reasons (Cowan et al 1981). The increased risk was, however, confined to premenopausal women, many of whom had received exogenous progestogen and the possibility cannot be excluded that administration of the hormone was responsible for the altered risk. The relative merits of these opposing hypotheses will be discussed further when the endocrinological actions of oestrogen and progestogen are considered.

Exogenous Hormones

Evidence for exogenous oestrogen alone or in combination with progestogen influencing risk of breast cancer comes from three sources

- (a) administration of contraceptive steroids to premenopausal women;
- (b) administration of hormones during pregnancy;
- (c) administration of hormones at and after the menopause.

(a) Oral Contraceptives: The influence of these preparations on risk of breast cancer has been studied extensively. In general, most case-control studies have failed to find a positive association between oral contraceptive use and risk (Vessey, 1984). However, it is possible that subgroups of women may have reason for concern such as those taking contraceptive steroids from an early age and for a long period of time and also those at already increased risk for other reasons. It has also been suggested by Pike et al. (1983) that contraceptive pills with high progestogenic potency are particularly associated with adverse risk. Whilst the study has been heavily criticised especially in terms of assessment of progestogenic potency, it draws attention to the problems of classifying all contraceptive steroids within the category of "the pill". A further complicating factor in assessing effects of oral contraceptives is that exogenous steroids are being administered against a background of normal ovarian steroid. It is conceivable therefore that the effects of contraceptive steroids on the breast could be either protective or deleterious depending on the nature and regularity of ovarian activity.

(b) Hormones during Pregnancy: particularly in the USA, very high doses of diethylstilboestrol were given to women during the 1950s and 1960s with the objective of preventing abortion and late pregnancy toxæmia. It is therefore doubly tragic that not only was the therapy ineffective but the treated women had an increased risk of breast cancer, although it has taken over 20 years for this to become apparent (Greenberg, 1984).

(c) Hormone Replacement at or after the Menopause: Postmenopausal-associated osteoporosis and other less debilitating effects of the menopause such as "hot flushes" and genital atrophy may be effectively treated by oestrogen replacement therapy (which usually involves the use of conjugated oestrogens). Whilst there is unequivocal evidence that such replacement therapy may cause endometrial cancer (Brunton, 1984), effects on breast cancer are much more controversial. Most, but not all, case-control studies suggest that oestrogen replacement carries with it an increased risk but this is only of a very modest size (Table 3). There also appears to be positive relationships between risk and dose of oestrogen and duration of use (Ross et al. 1980, Brinton et al. 1981, Hoover et al. 1981). It might be expected that, if replacement therapy is hazardous by increasing duration of exposure of the breast to oestrogen, administration to oophorectomized women would abolish the protective effects of early castration. No consensus exists as to whether this is so. Thus, whilst Brinton et al. (1981) reported a 50% increase in risk following use of menopausal hormones in oophorectomized women, Ross et al. (1980) could only find an increased risk in patients with intact ovaries. Effects of replacement oestrogen are clearly not restricted or concentrated in oophorectomized women. Oestrogen therapy may also be contra-indicated in certain groups of women such as those with a family history of breast cancer or an experience of previous benign breast disease. These results have led to the suggestion that breasts already prone to cancer for other reasons may be particularly susceptible to the effects of oestrogen.

In contrast to endometrial cancer in which the addition of progestogens reduces the hazards of oestrogen therapy (Gambrell 1982), the corresponding data for breast cancer is scanty. However, a recent study (Gambrell et al. 1983) has provided preliminary evidence that

the combination of progestogen to oestrogen may also decrease risk to breast cancer.

BIOCHEMICAL CONSIDERATIONS

Current dogma (Moolgavkar & Knudson 1981) suggests that breast cancers arise as a result of somatic mutations of DNA in undifferentiated breast stem cells (although whether these are of ductal or lobulo-alveolar origin is still in dispute). These errors in cell division are caused by initiating factors such as ionizing radiation and, perhaps, specific chemical carcinogens and viruses. Insofar as castration early in life gives marked protection against breast cancer, ovarian hormones must also have at least a permissive role in the development of the disease. These hormones are, however, not mutagenic in human systems and do not appear to damage nucleic acids. Their roles are therefore probably as promoters either to stimulate stem cell proliferation thereby increasing the population of cells susceptible to carcinogens or to encourage the selective growth of already transformed cells. The proliferative effects on the breast of oestrogen alone or in combination with progesterone would be compatible with such a mechanism of action. Furthermore the known risk factors, of early menarche, late menopause and exogenous administration of high doses of replacement oestrogen would also be in keeping with the promotion of breast cancer by the cumulative effects of excessive oestrogen. As a consequence of this concept, considerable work has been performed in an effort to demonstrate high levels of circulating oestrogen in patients with breast cancer and women at high risk to the disease. However, no consistent abnormality has been found in either urinary or plasma levels of oestrogens (Zumoff 1981, Forrest & Miller, 1985). A major problem in such

studies is that circulating levels of oestrogens do not necessarily reflect levels of oestrogens within the breast particularly in postmenopausal women. Indeed it may be pertinent that whilst circulating oestrogens fall markedly after the menopause, oestrogen within the breast is not significantly different in pre- and postmenopausal women. Furthermore whilst the major circulating oestrogens in postmenopausal women are oestrone and its sulphate (Samojlik et al. 1982), oestradiol tends to predominate within the breast (van Landeghem et al. 1985a). This is highly suggestive of local metabolism within the breast and, as has already been reviewed, evidence exists for metabolic pathways which would increase oestrogenicity within the breast including the conversion of oestrone sulphate to oestradiol and the transformation of androgens to oestrogens.

To put these effects into some kind of perspective, the risk factors linking exogenous and endogenous oestrogen excess with breast cancer are not as strong as the same factors in association with endometrial cancer. The breast must therefore be less susceptible to the tumour-promoting effects of oestrogens than the endometrium. This is supported by the larger amounts of oestrogen required to change the risk of breast cancer compared with that of endometrial cancer and the relative difficulty in demonstrating oestrogen receptor sites in normal breast compared with endometrium. Since the incidence rates of breast cancer are generally higher than those of endometrial cancer (Thomas 1984), either oestrogens play a lesser role in the genesis of breast cancer or the effects of oestrogens are subject to different modifying influences in the two tissues, e.g. oestrogen uptake, metabolism or interaction with other hormones is different. In any event, oestrogen would appear to be only one piece in the multifactorial jigsaw of breast cancer development.

Whether progesterone is another piece of the jigsaw is more debatable. The fundamental problem is to decide whether progestogens are co-promoters, oestrogen antagonists or neither. The case that progestogens may be hazardous is that, like oestrogens, they have proliferative properties in normal breast. The same risk factors which have been discussed in relation to oestrogen excess would therefore also favour promotional effects of progesterone. There is also undoubted synergism between oestrogen and progestogen (see Figure 6) and in most hormone-sensitive tissues the action of progesterone depends upon oestrogen. For example, the progestogen receptor protein which mediates the major action of progestogens is induced by oestrogen (Horowitz & McGuire, 1978).

Conversely the case for protective effects of progesterone is largely based on the epidemiological data that luteal insufficiency is associated with increased risk of breast cancer and that administration of progestogen to oestrogen during replacement therapy reduces risk of endometrial cancer and, perhaps, breast cancer. There is also some biochemical evidence that progestogens have antioestrogenic potential (Figure 6). Thus, in the uterus, progesterone blocks the production of oestrogen receptors (Hsueh et al. 1975). Furthermore in endometrium and breast cancers, progestogens induce the enzyme, oestrogen 17 β dehydrogenase, which catalyses the interconversion between oestradiol and oestrone. If, as a result, oestradiol is converted to oestrone, the oestrogenicity of the breast could be reduced. Equally, however, as the reaction is reversible, induction of enzyme activity could result in increased concentration of oestradiol. It seems possible that in endometrium the predominate direction is from oestradiol to oestrone (Tseng et al.

1981) whereas perfusion studies suggest that the reverse reaction is preferred in the breast (McNeill et al. 1985).

This highlights the difficulties in assessing the effects of both progestogens and oestrogens on the breast. Differences exist between the breast and other target organs not only in direct sensitivity to steroid hormones but in the fate of the hormones within the tissues. Thus, whereas cell division is apparently increased in the breast during the luteal phase of the cycle (Anderson and Ferguson, 1982), progesterone secreted from the corpus luteum inhibits mitosis in the endometrium (Novak and Woodruff, 1979). The distribution of oestrogen between cytoplasmic and nuclear compartments also differs completely in endometrial and breast cells (van Landeghem et al. 1985a). Similarly the application of observations from established breast cancers to the process of carcinogenesis is also fraught with dangers. Epithelial cells from malignant human breast tumours quite often have a different response to hormones than the normal mammary gland. For example the administration of high doses of oestrogen to postmenopausal women can lead to regression of cancerous growth whilst simultaneously stimulating proliferation of normal cells (Foote & Stewart, 1945). Furthermore, as the role of steroid hormones in carcinogenesis is probable not on differentiated normal cells but on undifferentiated stem cells or partially transformed cells, it is necessary to know the hormone sensitivity of the latter. A major handicap in assessing the potential effects of steroid hormones on induction of breast disease must therefore be the lack of suitable in vitro models for the developing and postmenopausal breast and the cells at risk to carcinogenic transformation within these tissues. Until answers to basic questions are available from these models, we

necessarily must be still in the realms of speculation and the therapeutic implications of steroid hormones will remain difficult to evaluate.

CONCLUSIONS

The evidence for the involvement of hormones in the development of the breast and its malignant tumours has been reviewed. Both oestrogen and progestogen have important and necessary roles to play in the natural development of the normal breast at puberty and during pregnancy. The involutionary changes occurring at and after the menopause result from diminished ovarian production of these hormones and can be reversed, at least in part, by exogenous administration of oestrogen. Prolonged and excessive oestrogen stimulation of the breast by delayed menopause occurring naturally or by hormone replacement therapy is associated with a small, but variable, increase in risk to breast cancer. The effects of progestogens are so controversial that it is unclear whether they are protective or hazardous to the breast. Only when more basic information is available from laboratory and clinical research will the exact benefits and dangers of steroid hormones become evident. This evidence will be useful not only for prevention of breast cancer but also for safe contraception and optimal treatment of menopausal disorders.

TABLE 1 INCIDENCE OF BREAST SECRETIONS AS OBTAINED BY NIPPLE
ASPIRATION

		% Secretors
Premenopausal Women		54.6
Parous	No Oral Contraceptive	97.5
	Current Oral Contraceptive	50.8
Nulliparous	No Oral Contraceptive	30.8
	Current Oral Contraceptive	26.4
Postmenopausal Women		37.4

TABLE 2 FACTORS ASSOCIATED WITH INCREASED RISK OF BREAST CANCER

Genetic : Family history of breast cancer

Environmental: Domicile in Western Europe and North American
industrial societies.

Heavy body build and high calorific diet.

Irradiation.

Hormonal : Female sex

Long menstrual life (early menarche, late menopause)

Anovulatory menstrual cycles

Nulliparity

Late age of first pregnancy

Prolonged high dose oestrogen therapy

(Previous benign breast disease?)

TABLE 3 RECENT CASE-CONTROL STUDIES OF RELATIVE RISK OF BREAST CANCER
IN WOMEN RECEIVING MENOPAUSAL OESTROGEN REPLACEMENT

<u>Reference</u>	<u>Relative Risk</u>	<u>Comment</u>
Sartwell et al. 1977	0.82	No relationship with duration of use.
Ross et al. 1980	1.1	Risk increased with dose and duration of use, particularly in women with intact ovaries.
Brinton et al. 1981	1.24	Risk increased with dose and duration of use, particularly in castrate women.
Hoover et al. 1981	1.4	Risk increased with dose and duration of use, particularly in women with a family history of breast cancer.
Kelsey et al. 1981	0.9	Risk not related to cumulative dose - few long-term users.
Hulka et al. 1982	1.7	Risk not related to dose or duration of use but associated with injectable oestrogen.
Sherman et al. 1983	0.6	Risk associated positively with body mass.
Nomura et al. 1986	0.7-1.1	Relative risk varies with type of controls. Risk increased in those with family history of breast cancer or a history of benign breast disease.

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LEGENDS TO FIGURES

Figure 1 (a) A group of normal resting lobules in loose fibrous tissue and fat, surrounding a terminal duct with secretions; nulliparous 18 years old woman. x 50, H & E.

(b) Fully developed lactational breast of a 27 year old woman, with expanded acinar structures separated by fibrous septae, sometimes containing ducts. x 50, H & E.

Figure 2 The multiplicity of hormones required for development of the breast depicted schematically from (a) a rudimentary prepubertal state to (b) the pubertal breast showing ductal elongation and ramification to (c) the adult breast with lobular-alveolar units and finally to (d) the pregnant breast showing full lobular development and multiple alveoli.

Figure 3 (a) Involuted lobules arranged around residual branched duct, surrounded by dense collagen. x 50, H & E.

(b) Postmenopausal involuted lobule in fibrous tissue and fat. x 50, H & E.

(c) Same lobule as 3 (b) at higher magnification to show prominent, paler outer myoepithelial layer (myoid atrophy) of ductules. x 160, H & E.

(d) Postmenopausal cystic lobular atrophy with adjacent duct showing prominent myoid layer at higher magnification. x 160, H & E.

Figure 4 Changes with age in the relative proportions of the principle elements of the breast.

Figure 5 (a) Persistent lobule in post menopausal breast, surrounded by fat. x 50, H & E.

(b) Higher magnification to show persistence of normal resting parenchymal architecture. x 160, H & E.

Figure 6 Interactions between oestrogen and progestogen which may be either synergistic to increase cell proliferation (cell on the left) or antagonistic to decrease cell proliferation (cell on the right).

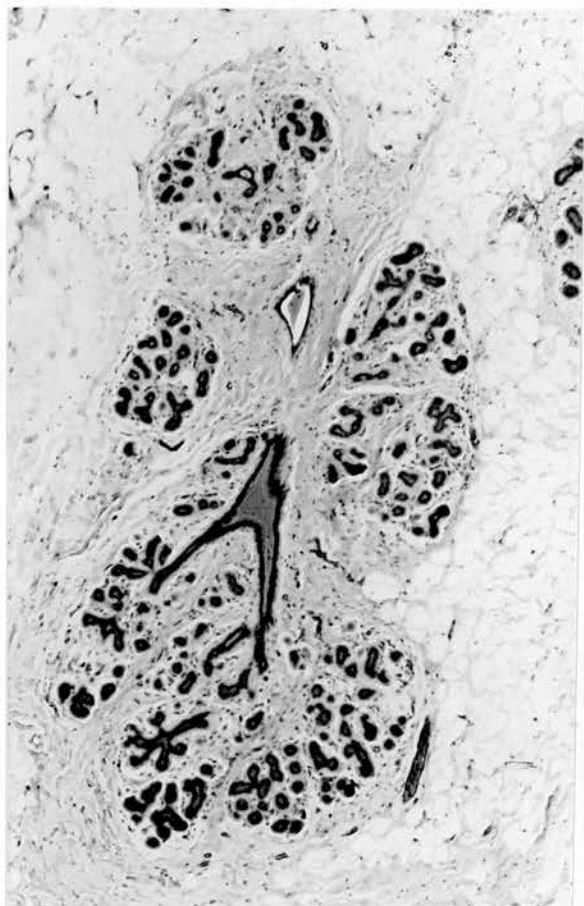


Figure 1a

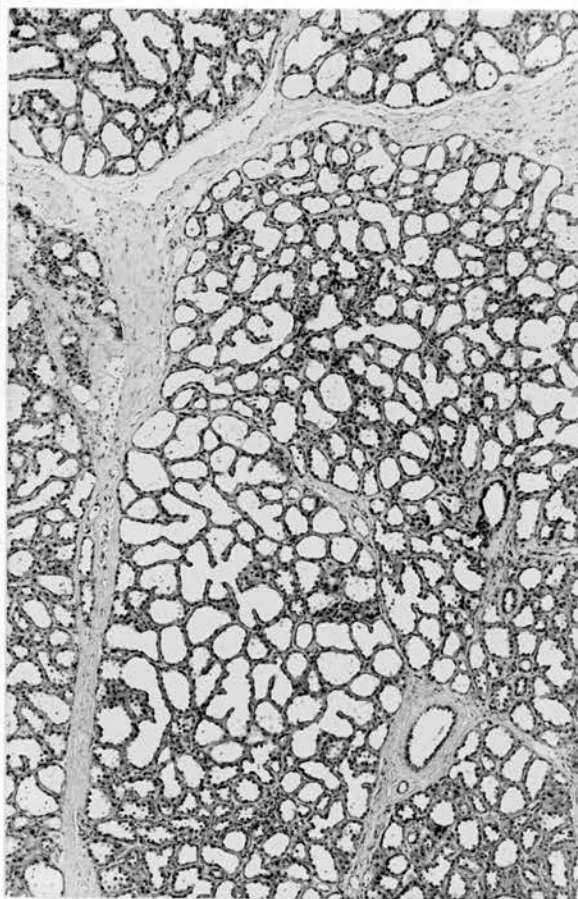


Figure 1b

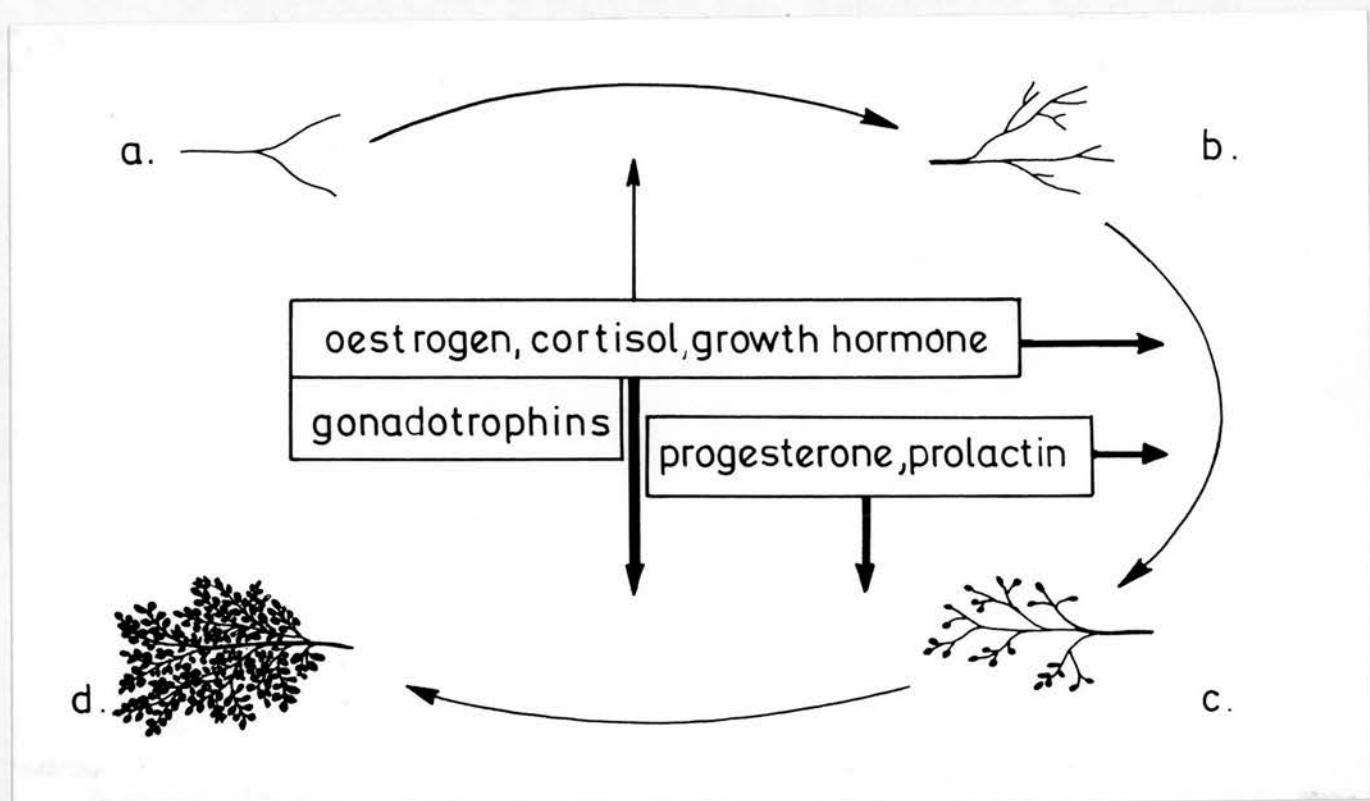


Figure 2



Figure 3a

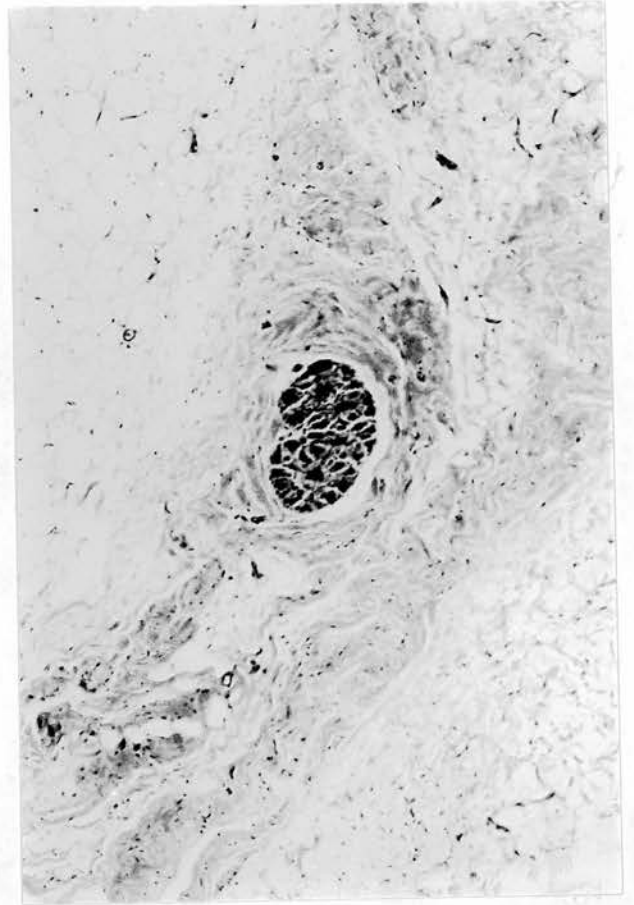


Figure 3b

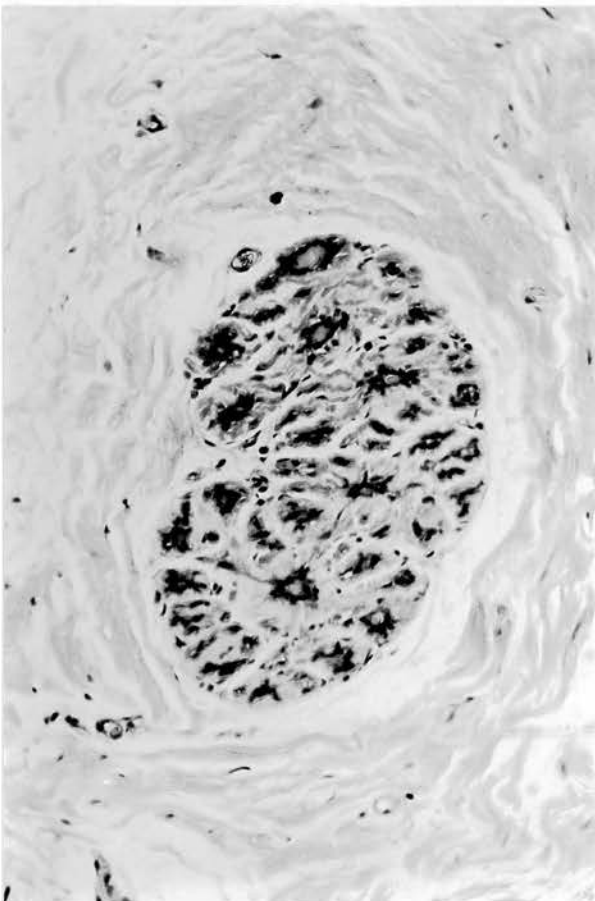


Figure 3c

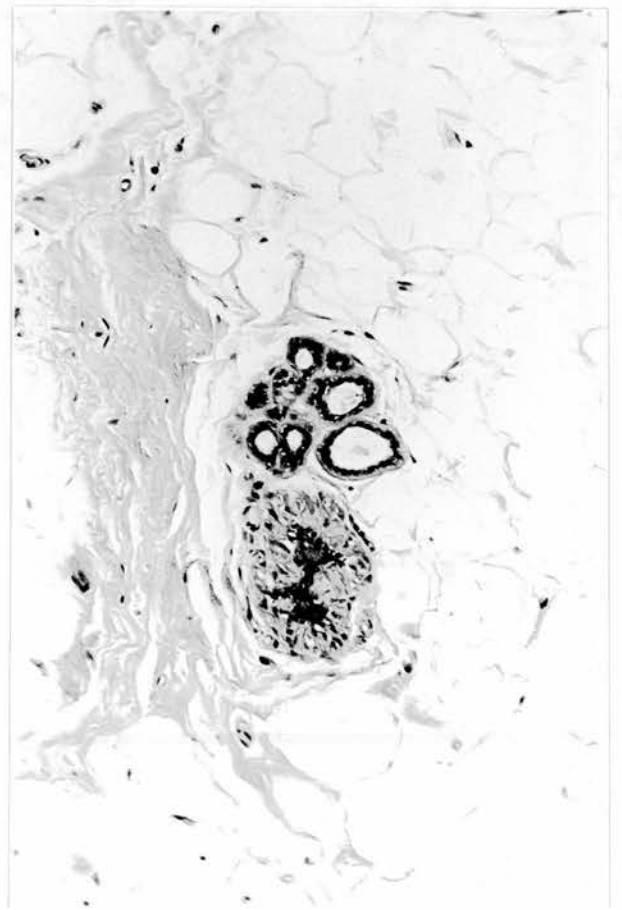


Figure 3d

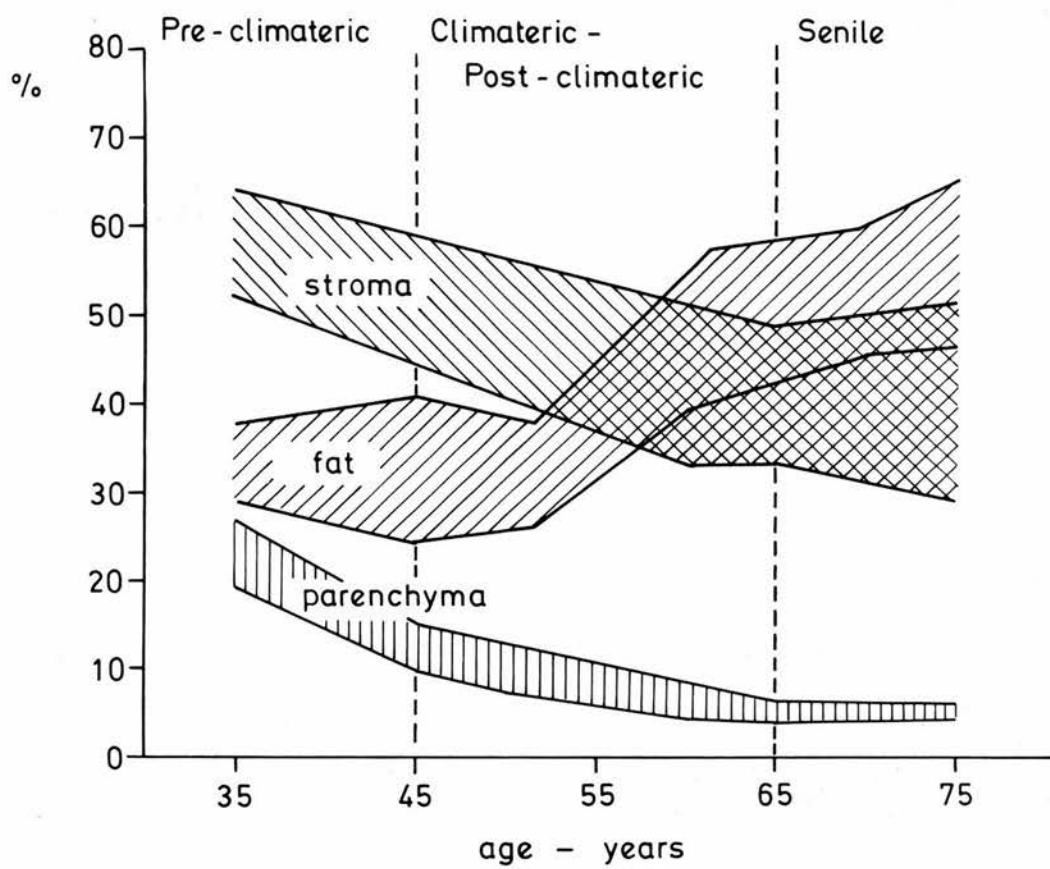


Figure 4

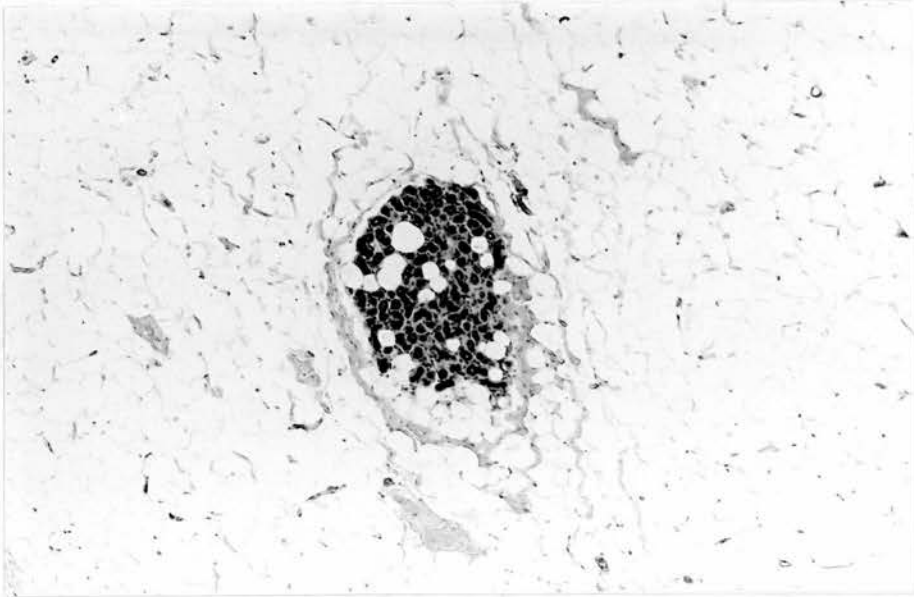


Figure 5a

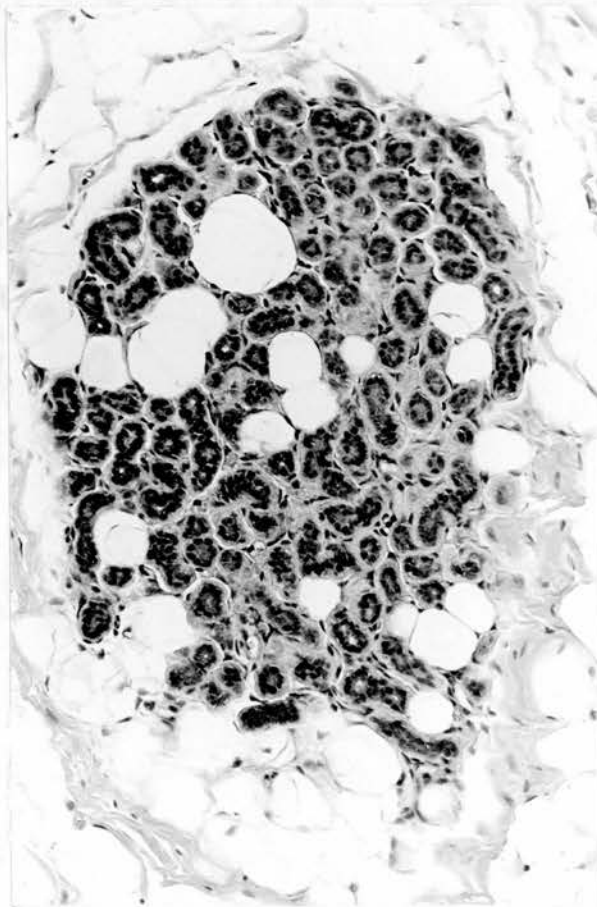
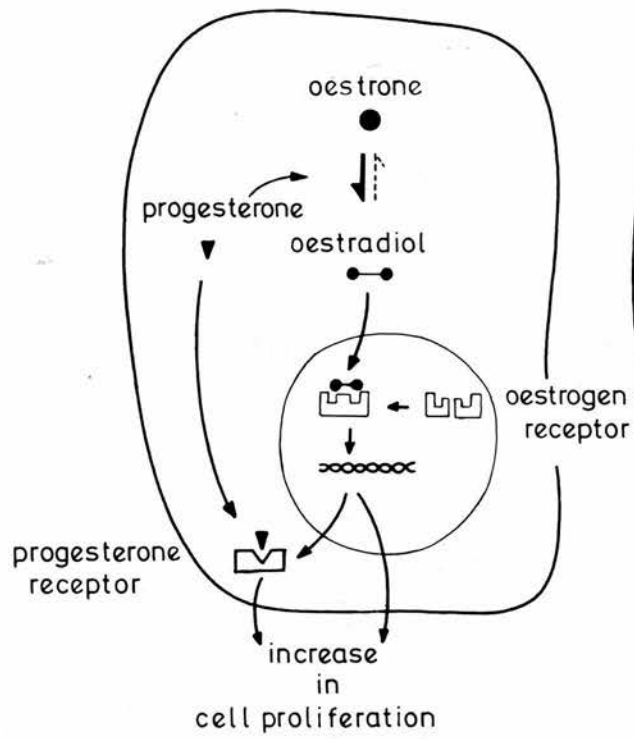


Figure 5b

Synergism of oestrogen and progestogen



Antagonism of oestrogen and progestogen

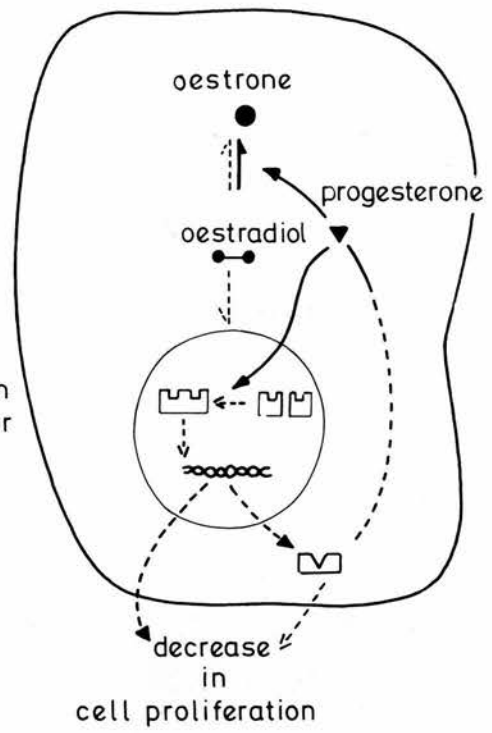


Figure 6